



Bandelow, B., Baldwin, D., Abelli, M., Bolea-Alamanac, B., Bourin, M., Chamberlain, S. R., Cinosi, E., Davies, S., Domschke, K., Fineberg, N., Grünblatt, E., Jarema, M., Kim, Y-K., Maron, E., Masdrakis, V., Mikova, O., Nutt, D., Pallanti, S., Pini, S., ... Riederer, P. (2017). Biological markers for anxiety disorders, OCD and PTSD: A consensus statement. Part II: Neurochemistry, neurophysiology and neurocognition. *World Journal of Biological Psychiatry*, 18(3), 162-214. <https://doi.org/10.1080/15622975.2016.1190867>

Peer reviewed version

Link to published version (if available):  
[10.1080/15622975.2016.1190867](https://doi.org/10.1080/15622975.2016.1190867)

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# Biological Markers for Anxiety Disorders, OCD and PTSD – A Consensus Statement – Part II: Neurochemistry, Neurophysiology and Neurocognition

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**World J Biol Psych in press, 2016**

**Authors' version of manuscript (may not be final version)**

Short Title: Biological markers for anxiety disorders

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## *Abstract*

**Objective:** Biomarkers are defined as anatomical, biochemical, or physiological traits that are specific to certain disorders or syndromes. The objective of this paper is to summarize the current knowledge of biomarkers for anxiety disorders, obsessive–compulsive disorder (OCD), and posttraumatic stress disorder (PTSD).

**Methods:** Findings in biomarker research were reviewed by a task force of international experts in the field, consisting of members of the World Federation of Societies for Biological Psychiatry (WFSBP) Task Force on Biological Markers and of the European College of Neuropsychopharmacology Anxiety Disorders Research Network (ADRN).

**Results:** The present article (Part II) summarizes findings on potential biomarkers in neurochemistry (neurotransmitters such as serotonin, norepinephrine, dopamine or GABA, neuropeptides such as cholecystokinin, neurokinins ANP, or oxytocin, the HPA axis, neurotrophic factors such as NGF and BDNF, immunology, and CO<sub>2</sub> hypersensitivity), neurophysiology (EEG, heart rate variability), and neurocognition. The accompanying paper (Part I) focuses on neuroimaging and genetics.

**Conclusion:** Although at present, none of the putative biomarkers is sufficient and specific as a diagnostic tool, an abundance of high quality research has accumulated that will improve our understanding of the neurobiological causes of anxiety disorders, OCD and PTSD.

**Keywords:** Anxiety disorders, neuroimaging, genetic, neurochemistry, neurobiology, review

**Funding:** None

## Abbreviations

Abbreviation	Explanation
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	Serotonin
5-HTP	Hydroxytryptophan
5-HTT	Serotonin transporter
5-HTTLPR	Serotonin-transporter-linked polymorphic region
A-SepAD	Adult Separation Anxiety Disorder
ACC	Anterior cingulate cortex
ACTH	Adrenocorticotrophic hormone or corticotropin
ADRN	Anxiety Disorders Research Network
ANP	Atrial natriuretic peptide
ASLO	Anti-streptolysin O
BDD	Body Dysmorphic Disorder
BDNF	Brain-derived neurotrophic factor
C-SepAD	Childhood Separation Anxiety Disorder
CBT	Cognitive-behavioural therapy
CCK	Cholecystokinin
COMT	Catechol-O-methyltransferase
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CSF	Cerebro-spinal fluid
DHEAS	Dehydroepiandrosterone sulfate
DAT	Dopamine transporter
DSM	Diagnostic and Statistical Manual of Mental Disorders
DST	Dexamethasone suppression test
ECNP	European College of Neuropsychopharmacology
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay
ERN	Error-related negativity
ERP	Event-related potential
fMRI	Functional magnetic resonance imaging
GABA	$\gamma$ -Aminobutyric acid
GABHS	Group A beta haemolytic streptococci
GAD	Generalized Anxiety Disorder
GWAS	Genome-wide association study
HF	High frequency (high frequency oscillation is a frequency-domain heart rate variability measure)
HPA axis	Hypothalamic-pituitary-adrenal axis
HPLC	High-performance liquid chromatography
HRV	Heart rate variability
IL	Interleukin
LF	Low frequency (low frequency oscillation is a frequency-domain heart rate variability measure)
MAO	Monoamine oxidase
MDD	Major Depressive Disorder
mPFC	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
NE	Norepinephrine (noradrenalin)
NET	Norepinephrine transporter
NGF	Nerve growth factor
NK	Neurokinin
OCD	Obsessive-Compulsive Disorder
OC-RD	Obsessive-Compulsive-Related Disorders
OFC	Orbitofrontal cortex
PANDAS	Pediatric autoimmune neuropsychiatric disorder associated with streptococcal infections
PANS	Pediatric acute-onset neuropsychiatric syndrome

PDA	Panic disorder with or without Agoraphobia
PFC	Prefrontal cortex
POMC	Proopiomelanocortin
PSG	Polysomnography
PTSD	Posttraumatic Stress Disorder
RMSSD	Root mean square of successive differences
SAD	Social Anxiety Disorder
SDNN	Standard deviation of normal sinus intervals
SNRI	Serotonin norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
SSRT	Stop signal reaction task
TNF	Tumor necrosis factor
TSPO	Translocator protein
WFSBP	World Federation of Societies for Biological Psychiatry

## Introduction

The present consensus statement of biological markers of anxiety disorders was organized by members of the WFSBP Task Force on Biological Markers and of the Anxiety Disorders Research Network (ADRN) within the European College of Neuropsychopharmacology Network Initiative (ECNP-NI) (Baldwin et al. 2010), an initiative intended to meet the goal of extending current understanding of the causes of central nervous system disorders, thereby contributing to improvements in clinical outcomes and reducing the associated societal burden.

The present article (Part II) summarizes the findings on potential biomarkers in neurochemistry, neurophysiology, and neurocognition. Part I (Bandelow et al. submitted) focuses on neuroimaging and genetics.

## Neurochemistry

The plasma appears to be a rational source for proteomic and metabolomic measurements in psychiatric conditions because it is easily accessible, and several molecules from the brain are transported across the blood-brain barrier and reach the circulation. However, it is difficult to draw conclusions from the neurochemical composition of plasma on the situation in brain cells. Spinal tap is an invasive method, and the composition of the cerebrospinal fluid (CSF) does not reflect exactly the neurochemistry in brain cells. Nevertheless, as a biomarker measure, such recourses are highly valuable, and several evidences in the literature points to

possible link between central nervous system and periphery. In the following sections, some of these findings are listed and described.

## *Neurotransmitters*

Monoaminergic systems have long been suggested to play a major role in depression and anxiety disorders. While the “reward system” is modulated by endogenous dopamine and opioids (Bandelow and Wedekind 2015; Barbano and Cador 2007; Berridge and Aldridge 2008; Le Merrer et al. 2009), the “punishment system” is mainly driven by serotonin (5-HT) (Daw et al. 2002; Stein 1971). Goal-directed behaviours are stimulated by dopamine and dopamine neurons have been suggested to be a substrate for intracranial self-stimulation (Aboitiz 2009; Mason and Angel 1984; Wise and Bozarth 1982). Norepinephrine (noradrenaline) has been connected to “emotional memory” and the consolidation and retrieval of the emotional arousal induced by particular behaviours (Goddard et al. 2010; van Praag et al. 1990).

### **Serotonergic system**

Findings on brain imaging and genetics of the serotonin system are summarized in Part I (Bandelow et al. submitted).

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine, which is found in the CNS, in blood platelets, and the gastrointestinal tract. The principal source of serotonin release in the brain are the raphe nuclei in the brainstem. It is hypothesized to have a dual role in aversive contingencies (Deakin 2013; Deakin and Graeff 1991). 5-HT can inhibit periaqueductal gray matter-mediated fight/flight responses from threats, while it can also facilitate amygdala-mediated anxiety responses. The latter mechanism has been demonstrated both in animals (Deakin 2013; Deakin and Graeff 1991) and humans (Blanchard et al. 2001; Feinstein et al. 2013; Mobbs et al. 2007). Such differences may explain partly the different types of emotions (Mobbs et al. 2007) and anxiety disorders seen in humans (Deakin and Graeff 1991). Therefore, reaction to threat, mediating periaqueductal-grey-mediated threats, related to the emotion named “fear”, may be more closely related with phobic, escape-dominant behavioural syndromes, such as specific phobias, social anxiety disorder (SAD) and panic disorder with or without agoraphobia (PDA) (Gray and McNaughton 2000; McNaughton and Corr 2004), while amygdala-mediated threats seem to be linked to the emotion named “anxiety” such as general anxiety disorder (GAD) and obsessive-compulsive disorder (OCD)



(Gray and McNaughton 2000; McNaughton and Corr 2004). Recently, 5-HT functional difference between fear and anxiety disorder was demonstrated using an acute tryptophan depletion technique that transiently lowers brain 5-HT (Corchs et al. 2015). In the study, the authors could demonstrate that decreasing the function of the 5-HT system, using tryptophan depletion, in patients in clinical remission leads to psychological and physiological exacerbation in response to stressors in PDA, SAD, and PTSD, while not in the GAD and OCD. This difference might be due to long-lasting neuronal changes, needed in anxiety disorders after serotonin-mediated therapeutics, in which acute 5-HT depletion does not cause such effects (Graeff and Zangrossi 2010). In the following paragraphs, the 5-HT involvement in the various disorders is discussed in more details.

### *PDA*

5-HT plasma levels measured by high-performance liquid chromatography (HPLC) were found to be significantly lower in PDA patients compared to control volunteers (Schneider et al. 1987b). Furthermore, in a study of males with PDA, serum 5-HT concentrations were measured via enzyme-linked immunosorbent assay (ELISA). The authors reported lower serum 5-HT in patients compared to control group at baseline, which was further decreased after treatment with the SSRI paroxetine, although symptom improvements were observed (Shutov and Bystrova 2008).

Platelet 5-HT binding was found to be decreased in PDA patients in two studies (Iny et al. 1994; Lewis et al. 1985), while most studies reported no difference comparing to controls (Butler et al. 1992; Innis et al. 1987; Norman et al. 1989a; Norman et al. 1989b; Nutt and Fraser 1987; Pecknold et al. 1987; Schneider et al. 1987a; Uhde et al. 1987). Moreover, platelet 5-HT concentration was reported also not to change in PDA patients (Balon et al. 1987; McIntyre et al. 1989), except one report, in which decreased 5-HT concentrations were observed (Evans et al. 1985). Two studies have reported increased platelet 5-HT uptake in PDA patients (Norman et al. 1986; Norman et al. 1989b), while two studies reported decreased platelet 5-HT uptake in panic disorder group compared to controls (Butler et al. 1992; Pecknold et al. 1988). Moreover, platelet aggregation in response to 5-HT was significantly lower in panic patients compared to controls (Butler et al. 1992).

CSF levels of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) were not different between PDA patients and healthy controls; nevertheless, in a small study with PDA patients responding to clomipramine or imipramine for at least 2 months, CSF 5-HIAA levels decreased significantly compared to baseline levels (Eriksson et al. 1991). Nevertheless, in

female major depressive disorder patients comorbid with PDA, CSF 5-HIAA levels were significantly higher than in major depressive patients without PDA and healthy volunteers (Sullivan et al. 2006). Higher CSF 5-HIAA in women with comorbid major depressive disorder and lifetime panic disorder is indicative of greater 5-HT release, increased 5-HT metabolism, and/or decreased 5-HIAA clearance in this group. Esler and colleagues (2004) measured brain 5-HT turnover via measurement of 5-HIAA levels in plasma from internal jugular veins that has a direct overflow from brain neurons and not from the cerebrovascular sympathetic nerves (Lambert et al. 1995). A significant increase in brain 5-HT turnover, estimated from the jugular venous overflow of 5-HIAA, was observed in non-medicated PDA patients compared with healthy subjects (Esler et al. 2004).

Another approach measuring 5-HT disruption is via measurement of antibodies directed at the 5-HT system, such as anti-serotonin and 5-HT anti-idiotypic antibodies (directed at the serotonin receptors). Using this approach, Coplan et al. (1999) could show significantly elevated levels of plasma anti-serotonin and serotonin anti-idiotypic antibodies in panic disorder patients compared to controls. These findings suggest an autoimmune mechanism interrupting the 5-HT system in PDA.

### *GAD*

Platelet 5-HT binding was found to be decreased in GAD patients (Iny et al. 1994). 5-HT binding in lymphocytes did not differ in GAD patients compared to controls (Hernandez et al. 2002). Moreover, both 5-HT and 5-HIAA in platelet-rich and -poor plasma as well as lymphocytes did not differ between GAD patients and controls (Hernandez et al. 2002).

### *SAD*

The therapeutic efficacy of SSRIs or SNRIs strongly suggests that 5-HT plays a crucial role in SAD. Patients with SAD show exaggerated cortisol response to the serotonin-releasing compound fenfluramine, indicating supersensitivity of the post-synaptic serotonin receptors (Tancer 1993). In a similar study, SAD patients received challenges for serotonergic (fenfluramine), dopaminergic (levodopa), and noradrenergic (clonidine) systems in a double-blind study. They had an increased cortisol response to fenfluramine administration, compared with healthy volunteers. Neither the prolactin response to fenfluramine, the growth hormone or norepinephrine response to clonidine, nor the prolactin or eye-blink responses to levodopa, differed between patients with SAD and healthy volunteers (Tancer et al. 1994b).

Platelet 5-HT<sub>2</sub> receptor density did not differentiate between the SAD patients and controls, but was associated with severity (Chatterjee et al. 1997).

Patients with SAD, healthy control subjects, and OCD control subjects were challenged with single doses of the partial serotonin agonist oral m-chlorophenylpiperazine (m-CPP) and placebo. SAD patients did not significantly differ from normal or OCD control subjects in prolactin response to m-CPP. Female patients with SAD had more robust cortisol responses to the m-CPP challenge (Hollander et al. 1998).

SAD patients, who were successfully treated with an SSRI, underwent a tryptophan depletion challenge combined with a public speaking task. Salivary alpha-amylase, a marker of autonomic nervous system response, and HPA-axis response, as measured with salivary cortisol, were assessed. The tryptophan depletion group showed a significant larger salivary alpha-amylase response to the public speaking task as compared to the placebo group, whereas no differences were seen in cortisol responses (van Veen et al. 2009).

### *OCD*

The measuring of peripheral serotonergic parameters, like whole-blood 5-HT concentration, CSF concentration, platelet 5-HT transporter (5-HTT), 5-HT<sub>2A</sub> receptor binding characteristics and platelet inositol 1,4,5-triphosphate content, is the oldest classical approach, which has identified some predictors of clinical outcome of the treatment in OCD patients medicated with SSRIs.

In an early study, Thoren et al. (1980) showed initially elevated 5-HIAA levels in the CSF and a decrease during treatment were associated with better clinical outcome in patients treated with clomipramine (Flament et al. 1985).

There was no difference in blood 5-HT content between children and adolescents with severe OCD and the normal controls. However, OCD patients with a family history of OCD had significantly higher blood 5-HT levels than did either the OCD patients without family history or the healthy controls (Hanna et al. 1991). Blood 5-HT levels were decreased after treatment with SSRIs (Humble et al. 2001; Humble and Wistedt 1992; Kremer et al. 1990), and higher 5-HT concentrations were associated with better outcome after treatment of OCD (Aymard et al. 1994; Delorme et al. 2004).

Serotonin platelet binding capacity was found to be reduced in children and adolescents with OCD, but not in Tourette syndrome (Sallee et al. 1996). The binding capacity of the 5-HTT for SSRIs and the tricyclic antidepressant imipramine decreased in untreated OCD patients

(Marazziti et al. 1996; Sallee et al. 1996). After treatment with the tricyclic antidepressant clomipramine, binding was decreased (Black et al. 1990), whereas another study has found increased binding after treatment with the SSRI with fluvoxamine and or clomipramine. (Marazziti et al. 1992).

### *PTSD*

In an early review of trauma-related studies involving epinephrine, norepinephrine, and serotonin, evidence of serotonergic dysregulation in PTSD was reported, including frequent symptoms of aggression, impulsivity, depression and suicidality, decreased platelet paroxetine binding, blunted prolactin response to fenfluramine, exaggerated reactivity to m-chloro-phenyl-piperazine (mCPP), and clinical efficacy of SSRIs (Southwick et al. 1999).

No change in 5-HT<sub>1A</sub> receptor binding was found in a study by Bonne et al. (2005). Lower number of platelet {<sup>3</sup>H}-paroxetine binding sites and a lower dissociation constant for {<sup>3</sup>H}-paroxetine binding in combat veterans with PTSD compared to normal control subjects was reported (Fichtner et al. 1995). Platelet 5-HT concentration was significantly lower in suicidal PTSD and non-PTSD patients compared to non-suicidal patients or healthy controls (Kovacic et al. 2008). Compared with the control subjects, the PTSD patients showed significantly lower platelet-poor plasma 5-HT levels, elevated platelet-poor plasma norepinephrine levels, and significantly higher mean 24-hour urinary excretion of all three catecholamines (norepinephrine, dopamine, and HVA) (Spivak et al. 1999).

During presentation of a trauma-related video, CSF concentrations of 5-HIAA diminished, but there was only a trend for statistical significance of this finding (Geraciotti et al. 2013).

### **Dopaminergic system**

Dopamine is a neurotransmitter, which is involved in reward-motivated behaviour and motor control. Findings on brain imaging and genetics of the dopamine system are summarized in Part I (Bandelow et al. submitted). Similarly as for the serotonergic system, in the following paragraph the current findings related to the dopaminergic system are described.

### *PDA*

Eriksson et al. (1991) reported no significant change in CSF levels of HVA, the major metabolite of dopamine in patients with PDA compared to healthy controls. Nevertheless, another study in both PDA and SAD, low CSF HVA levels were observed (Johnson et al. 1994).

### *SAD*

In a study evaluating eye-blink response to administered levodopa, no dysfunction of the dopaminergic system has been reported (Tancer et al. 1994a).

### *PTSD*

In the aforementioned study by Geraciotti (2013), CSF HVA concentrations diminished significantly after the traumatic video. Compared with control subjects, PTSD subjects showed significantly higher mean 24-hour urinary excretion of dopamine (Spivak et al. 1999).

## **Noradrenergic system**

Norepinephrine (noradrenaline; NE) is a catecholamine produced mainly in the locus coeruleus in the pons. It is an important neurotransmitter in the autonomic nervous system. The metabolism and functions of norepinephrine have been studied in extent in depression and anxiety disorders. A hypofunction is postulated for the former, a hyperfunction for the latter. Findings on brain imaging and genetics of the noradrenergic system are summarized in Part I (Bandelow et al. submitted).

### *PDA*

Stimulation of noradrenergic systems produces abnormal changes in measures of anxiety, somatic symptoms, blood pressure and plasma NE metabolite and cortisol levels in patients with PDA but not in patients with GAD, OCD, depression or schizophrenia, indicating specificity of abnormality in the regulation of the NE system in patients with PDA (Boulenger and Uhde 1982; Heninger and Charney 1988).

There is a body of evidence for NE involvement in anxiety in humans; e.g. anxiety can be induced using NE neuronal activators such as piperoxane and yohimbine (Redmond and Huang 1979). In patients with PDA, peripheral markers, including platelet aggregation to NE and to 5-HT, platelet  $\alpha_2$ -receptor density, lymphocyte  $\beta$ -receptor density,  $\{^3\text{H}\}$ -ketanserin binding to platelet 5-HT<sub>2</sub> receptors and  $\{^3\text{H}\}$ -serotonin-transporter uptake into platelets, largely remained abnormal during six months treatment with either clomipramine or lofepramine suggest that, despite clinical improvement (Butler et al. 1992). Therefore, these peripheral markers have been suggested to be trait markers in patients with PDA. Adrenergic receptor function has been measured in several clinical studies. Platelet  $\alpha_2$ -adrenoceptors have been studied in PDA patients using clonidine and yohimbine binding assays and correlated to symptom ratings and measurement of lying and standing plasma adrenaline and NE levels

(Cameron et al. 1996). Tritiated clonidine binding was decreased and lying heart rate was increased in PDA patients before treatment (fluoxetine, tricyclics or alprazolam). The magnitude of decrease in receptor binding was correlated with symptom severity and standing plasma NE (Cameron et al. 1996). In a similar approach, Gurguis et al. (1999) showed that patients with PDA had high  $\alpha_2$ -adrenoceptor density in both conformational states.

Stimulation of the locus coeruleus, an area containing most of the noradrenergic cell bodies of the brain, has been shown to induce anxiety and to raise the concentration of the main central NE metabolite, 3-methoxy-4-hydroxyphenyl glycol (MHPG) in patients with panic attacks; the decrease in plasma MHPG concentrations was found to parallel the response of patients with PDA to treatment (Charney et al. 1983). However, this could not be confirmed in a study of the effects of imipramine in PDA by Nutt and Glue (1991). Similarly, CSF levels of MHPG were not changed significantly in patients with PDA (Eriksson et al. 1991). On the other hand, Lista (1989) reported short time urine sampling to measure NE excretion as a marker for monitoring sympathetic activity. Norepinephrine excretion was highest in major depression, followed by “minor” depression, anxiety disorders and healthy controls. Although plasma catecholamines (NE and epinephrine), blood pressure and heart rate were only partially found to be statistically significantly correlated with salivary  $\alpha$ -amylases, Kang (2010) proposed  $\alpha$ -amylase as a measure of stress sensitivity causing an increase in anxiety scores. Recently, it was shown that epinephrine (24 hour urine collection) was positively correlated with anxiety but not with depression, whereas 24-hour urinary NE excretion was neither correlated with anxiety nor depression (Paine et al. 2015).

A low pre-treatment  $\beta$ -adrenoceptor affinity was found to predict the treatment response to paroxetine in patients with PDA and was suggested as a biomarker of pharmacological outcome in PDA (Lee et al. 2008).

### *PTSD*

Compared with control subjects, PTSD patients showed significantly elevated platelet-poor plasma NE levels, and significantly higher mean 24-hour urinary excretion of all three catecholamines (NE, dopamine, and HVA) (Spivak et al. 1999).

### **Gamma-aminobutyric acid (GABA)**

There is ample evidence that the pathogenesis of anxiety disorders is in part linked to a dysfunction of central inhibitory mechanisms. With regard to neurotransmission, the gamma-

amino-butyric acid (GABA) system serves as the most important inhibitory neurotransmitter system (Domschke and Zwanzger 2008). According to both preclinical and clinical studies, this system has been suggested to be strongly involved in the pathophysiology of anxiety and anxiety disorders. For example, benzodiazepines, which act at the GABA system, are used to treat anxiety. GABA is synthesized by a specific enzyme, glutamate acid decarboxylase (GAD) from glutamate. Released in the synaptic cleft, it either binds on GABA receptors or is removed by the main degradative enzyme GABA-transaminase (GABA-T) (for a review, see Olson, 2002).

So far, three major subtypes of GABA receptors have been identified: GABA<sub>A</sub>, GABA<sub>B</sub> and GABA<sub>C</sub> receptors. GABA<sub>A</sub> and C receptors belong the class of ligand-gated ion channels, GABA<sub>B</sub> receptors serve as transmembrane receptors, coupled with G-proteins and activate second messenger systems (Chebib and Johnston 1999). However, the fast inhibitory action of the neurotransmitter GABA is mediated through GABA<sub>A</sub> receptors. A large variety of GABA<sub>A</sub> receptor subtypes has been characterized so far:  $\alpha$  1-6,  $\beta$  1-3,  $\gamma$  1-3,  $\delta$ ,  $\epsilon$  1-3,  $\theta$ ,  $\pi$  (Jacob et al. 2008); see Figure 1.

Insert Figure 1

GABA<sub>A</sub> receptors consist of two  $\alpha$  subunits, two  $\beta$  subunits and one  $\gamma$  or  $\delta$  subunit (Jacob et al. 2008). Moreover, there are two distinct binding sites on the GABA<sub>A</sub> receptor: whereas GABA itself binds on the GABA binding side, which is located at the interface between the  $\alpha$  and  $\gamma$  subunit, anxiolytic agents such as benzodiazepines bind at the benzodiazepine binding site at the interface between the  $\alpha$  and the  $\gamma$  subunit. According to several preclinical studies, anxiolytic effects of benzodiazepines have been shown to be mostly mediated by the  $\alpha_2$ -subunit of the GABA<sub>A</sub> receptor (Low et al. 2000).

Therefore, also a specific role of distinct GABA<sub>A</sub> receptor subunits can be hypothesized with regard to the pathogenesis of anxiety. However, research on specific subunit selective psychopharmacological compounds targeting the  $\alpha_2$ -subunit of the GABA<sub>A</sub> receptor and lacking sedative or other associated side effects of benzodiazepines is still underway.

## *PDA*

### Neurochemistry

An interesting approach investigating the role of GABA<sub>A</sub> receptors on the pathogenesis of panic attacks stems from Nutt and colleagues (1990) who suggested alterations in benzodiazepine receptor sensitivity in patients with PDA. After intravenous challenge, subjects suffering from panic disorder exhibited panic attacks after flumazenil injection, a phenomenon which has been interpreted as a possible shift of the “receptor setpoint” (Nutt et al. 1990). However, these results have not been replicated so far (Ströhle et al. 1999).

There is also evidence for a dysfunction of GABA<sub>A</sub> receptor modulatory neuroactive steroid regulation in panic disorder patients (Rupprecht 2003). It has been demonstrated that panic disorder patients show increased concentrations of GABA agonistic 3 $\alpha$ -reduced neuroactive steroids (Ströhle et al. 2002), which has been interpreted as a counter-regulatory mechanism against the occurrence of spontaneous panic attacks. In contrast, during experimentally induced panic induction with lactate or cholecystokinin-tetrapeptide (CCK-4) panic disorder patients show a significant decrease of GABA agonistic 3 $\alpha$ -reduced neurosteroids along with an increase of the antagonistic 3 $\alpha$ -reduced isomer compared with healthy controls (Ströhle et al. 2003).

Translocator protein (TSPO) is a 18 kilodalton protein in the mitochondrial membrane, which was first thought to be a peripheral binding site for benzodiazepines. However, recent research has found that it is not only expressed in the body but also in the brain. Ligands of this protein may promote the synthesis of endogenous neurosteroids. Some metabolites of progesterone are potent, positive allosteric modulators of  $\gamma$ -aminobutyric acid type A receptors. Their concentrations are reduced during panic attacks in patients with PDA (Ströhle et al. 2003). Unexpectedly, patients with PDA had significantly greater concentrations of the agonistic 3 $\alpha$ -reduced neuroactive steroids (Ströhle et al. 2002). The translocator protein ligand XBD173 enhanced GABA-mediated neurotransmission and exerted antipanic activity in humans. In contrast to benzodiazepines, the drug did not cause withdrawal symptoms or sedation. Thus, translocator protein ligands are promising candidates for novel anxiolytic drugs (Rupprecht et al. 2009).



Neuroimaging studies found a reduction of GABA concentrations and benzodiazepine binding in patients with PDA (see chapter Neuroimaging, Part I, (Bandelow et al. submitted). A few genetic studies tried to elucidate role of GABA in anxiety disorders (see chapter Genetics, Part I (Bandelow et al. submitted).

#### Pharmacological modulation of the GABA system

From a clinical point in view, the significance of the GABA system in the pathophysiology of panic and anxiety has also been derived from beneficial effects on clinical symptoms following selective GABAergic treatment. Apart from the rapid and strong anxiolytic properties of benzodiazepines, targeting the benzodiazepine binding site of the GABA<sub>A</sub> receptor also modulation of GABA metabolism has been shown to reduce anxiety and the occurrence of panic attacks. Among anticonvulsants tiagabine and vigabatrin both increase GABA availability via a reduction of GABA degradation by inhibition of the GABA transaminase (vigabatrin) or the inhibition of GABA reuptake via blockade of the GABA transporter GAT-I (tiagabine). For both compounds, anxiolytic action has been suggested in clinical studies and studies using pharmacological panic induction with cholecystokinin-tetrapeptide (for a review, see Zwanzger & Rupprecht, 2005 ).

Also, other drugs that enhance GABAergic tone (e.g. barbiturates, ethanol, valproate) have anxiolytic effects, while negative modulators produce anxiogenic-like effects (Kalueff and Nutt 2007; Zwanzger et al. 2001; Zwanzger et al. 2009).

#### *SepAD and benzodiazepines*

Several studies favour the role of the TSPO as a useful biological marker of A-SepAD. The TSPO is involved in the secretion of neurosteroids, whose levels are reported to be changed in several diseases and to be implicated in the pathogenic mechanisms of anxiety and mood disorders in humans. A reduction of platelet expression of TSPO density was found to relate specifically to the presence of A-SepAD in samples of patients with PDA (Pini et al. 2005) or major depression (Chelli et al. 2008) or bipolar depression (Abelli et al. 2010). Furthermore, Costa et al. (2012) found Ala147Thr substitution in TSPO to be associated with A-SepAD in patients with depression.

## *Neuropeptides*

### **Cholecystokinin (CCK)**

Cholecystokinin is one of the most abundant neurotransmitter peptides in the brain and has been shown to induce excitation of central neurons as well as inhibitory postsynaptic effects (Bourin and Dailly 2004). CCK-1 and 2 receptors (G protein-coupled receptors) (recently reclassified as A and B) are widely distributed throughout the CNS. A large body of evidence suggests that the neuropeptide CCK might be an important modulator of the neuronal networks that are involved in anxiety, in particular in PDA.

#### *PDA*

In humans, CCK-induced anxiety may be mediated via CCK-B receptors (vs. CCK-B and A in mice) (Li et al. 2013). Intravenous administration of exogenous CCK-4, CCK-8 or the CCK agonist pentagastrin produced panic-like attacks in healthy volunteers within one minute, and these effects were attenuated by pretreatment with benzodiazepines (Bradwejn et al. 1991b; de Montigny 1989). The most common clinical effects observed after administration of intravenous CCK-4 were dyspnea, palpitations/tachycardia, chest pain/discomfort, faintness, dizziness, paresthesia, hot flushes/cold chills, nausea/ abdominal distress, anxiety/fear/apprehension and fear of losing control – a cluster of symptoms similar to those observed in spontaneous panic attacks in PDA.

In addition, the dose-response to intravenous CCK-4, reliably differentiates PDA patients from healthy controls with no personal or family history of panic attacks (Bradwejn et al. 1992). Furthermore, an effect-dose ranging was found in healthy volunteers (Bradwejn et al. 1991a). While the panic rate after injection of 25 µg of CCK-4, was 91% for patients as compared to only 17% for controls; 50 µg induced a full-blown panic attack in 100% of patients vs. 47% of controls.

In contrast to the findings in patients with PDA, in CCK-4-sensitive healthy volunteers, treatment with an antipanic SSRI did not cause a reduction of CCK-4-induced panic attacks beyond the effect of placebo (Toru et al. 2013). However, significant reduction of CCK-induced anxiety was observed after administration of the benzodiazepine alprazolam and the GABAergic anticonvulsant vigabatrin (Zwanzger et al. 2001; Zwanzger et al. 2003). Baseline anxiety is not a major determinant of the subjective panic response to CCK-4, emphasizing the importance of neurobiological factors (Eser et al. 2008). It was proposed that

benzodiazepine-mediated antagonism of CCK-induced excitation might be an important mechanism by which benzodiazepines exert their clinically relevant actions.

Moreover, in PDA patients, decreased concentrations of CCK-8 in the CSF have been reported as compared to control subjects (Lydiard et al. 1992). Concentrations of CCK-8 in lymphocytes were also significantly reduced in patients with PDA compared with healthy controls (Brambilla et al. 1993). Finally, CCK-B receptor expression and binding are increased in animal models of anxiety. These findings are in favour of abnormalities in the CCK system in PDA patients.

The key regions of the fear network, such as basolateral amygdala (Del Boca et al. 2012), hypothalamus, periaqueductal grey, or cortical regions such as the anterior cingulate cortex seem to be connected by CCK-ergic pathways (Dieler et al. 2008). Moreover, these effects seem to be modulated by molecular mechanisms, since neurochemical alterations were dependent on neuropeptide S genotype (Ruland et al. 2015). In humans, amygdala activation may be involved in the subjective perception of CCK-4-induced fear (Eser et al. 2009). More recent work suggests that, in the amygdala, CCK may act in concordance with the endogenous cannabinoid system in the modulation of fear inhibition and extinction (for review, see (Bowers et al. 2012). In addition, CCK-4-induced panic is accompanied by a significant glutamate increase in the bilateral anterior cingulate cortex (ACC) (for review, see Bowers et al., 2012). In contrast to placebo, alprazolam abolished the activation of the rostral ACC after challenge with CCK-4 and increased functional connectivity between the rostral ACC and other anxiety-related brain regions such as the amygdala and the prefrontal cortex. Moreover, the reduction in the CCK-4 induced activation of the rostral ACC correlated with the anxiolytic effect of alprazolam (Leicht et al. 2013). Finally, social stress-induced behavioural deficits are mediated partly by CCK-B receptors as a molecular target of  $\Delta$ FosB in the medial prefrontal cortex (mPFC) and by molecular adaptations in the mPFC involving  $\Delta$ FosB and CCK through cortical projections to distinct subcortical targets. In fact, CCK in mPFC-basolateral amygdala projections mediates anxiety symptoms (Vialou et al. 2014).

CCK also interacts with several anxiety-relevant neurotransmitters such as the serotonergic, GABAergic and noradrenergic systems, as well as with endocannabinoids, neuropeptides Y and S; for a review, see Zwanzger et al., (Zwanzger et al.). For a review of CCK genes in anxiety disorders, see Part I (Bandelow et al. submitted).

In conclusion, experimental panic induction with CCK-4 has been established as a model to study the pathophysiology of PDA and might serve as a tool to assess the anti-panic potential

of novel anxiolytic compounds if the challenge procedure is carried out according to strictly comparable conditions (Eser et al. 2007).

### **Neurokinins (tachykinins)**

Central neurokinins (tachykinins) have been shown to play a role in the modulation of stress-related behaviours and anxiety. Different forms exist, termed neurokinins 1, 2 and 3.

Substance P, a ligand of the neurokinin 1 (NK<sub>1</sub>) receptor, is released in response to stress, anxiety, and pain (Carrasco and Van de Kar 2003; Ebner and Singewald 2006; Saria 1999).

#### *PDA*

In a positron emission tomography (PET) study, decreased neurokinin (NK<sub>1</sub>) receptor binding was found in patients with PDA (Fujimura et al. 2009); see Part I (Bandelow et al. submitted). It was tried to develop neurokinin antagonists for the treatment of anxiety disorder. The NK<sub>1</sub> receptor antagonist *vestipitant* showed anxiolytic effects in a preliminary study (Poma et al. 2014). However, *vofopitant*, a NK<sub>1</sub> antagonist, and *onasetant*, a NK<sub>3</sub>-receptor antagonist, were not effective (Kronenberg et al. 2005; Poma et al. 2014).

#### *Specific phobia*

In a PET study with women with specific phobias, the uptake of the labeled NK<sub>1</sub> receptor antagonist [<sup>11</sup>C]GR205171 was significantly reduced in the right amygdala during phobic stimulation (Michelgard et al. 2007).

### **Atrial natriuretic peptide**

#### *PDA*

Atrial natriuretic peptide (ANP) is not only synthesized by atrial myocytes and released in the circulation (de Bold 1985), but is also found in different brain areas where specific receptors have been found. ANP has been shown to inhibit the CRH-stimulated release of adrenocorticotrophic hormone (ACTH) (Kellner et al. 1992) and cortisol (Ströhle et al. 1998a). Also, peripheral and central administration of ANP has an anxiolytic activity in different animal models of anxiety (Ströhle et al. 1997). In patients with PDA, ANP reduced CCK-4-induced panic attacks (Ströhle et al. 2001) and an activation of the HPA system (Wiedemann et al. 2001). Furthermore, a significantly accelerated ANP release has been described in patients with lactate-induced panic attacks (Kellner et al. 1995), and it has been suggested that this increase also contributes to the paradoxical blunting of ACTH and cortisol secretion

during lactate-induced and possibly spontaneous panic attacks. As physical activity increases ANP concentrations, it has been suggested that the anxiolytic activity of exercise might be associated with increased ANP concentrations. And indeed, the anxiolytic activity of a single exercise bout was correlated with the increased ANP concentrations (Ströhle et al. 2006).

Although there have been major efforts to develop small-molecule, non-peptide receptor ligands acting as CRH<sub>1</sub> antagonists, NK-antagonists or ANP agonists, we still lack convincing clinical proof-of-concept studies with peptidergic treatment approaches in patients with anxiety disorders.

## **Oxytocin**

### *SAD*

In humans, the anxiety modulation of oxytocin has been demonstrated by showing reduced amygdala responses to aversive stimuli. Moreover, intranasal oxytocin promotes trust, and reduces the level of anxiety, possibly at the level of the amygdala (Heinrichs et al. 2009; Kirsch et al. 2005; Kosfeld et al. 2005; Zak et al. 2005). The dysregulation of oxytocin as a putative mechanism underlying social attachment has been examined widely in animal studies, e.g. (Williams et al. 1994), and recently has become of interest in human studies.

In a study examining oxytocin as add-on to exposure therapy in patients with SAD, participants administered with oxytocin showed improved positive evaluations of appearance and speech performance, but these effects did not generalize to improve overall treatment outcome from exposure therapy (Guastella et al. 2009).

A role of oxytocin in SAD has also been shown in neuroimaging studies (chapter Neuroimaging, Part I (Bandelow et al. submitted)).

### *SepAD*

Genetic studies have shown a possible role of oxytocin in SePAD (chapter Genetics, Part I, (Bandelow et al. submitted)).

### *PTSD*

In Vietnam veterans with PTSD, no beneficial effects of intranasal oxytocin on physiological responses to combat imagery were observed (Pitman et al. 1993).

## *Hypothalamic-pituitary-adrenal (HPA) axis*

### **PDA**

There has been a growing number of studies aiming to delineate the possible role of HPA axis function in the pathophysiology of the anxiety disorders, mainly through the use of plasma, urine, or saliva cortisol levels in basal conditions or after pharmacological or psychological challenge test as a potential biological marker (Elnazer and Baldwin 2014).

#### *Basal levels*

Baseline plasma levels of cortisol in PDA patients were reported to be elevated during the day (Goetz et al. 1989; Nesse et al. 1984; Roy-Byrne et al. 1986) or during the night (Abelson et al. 1996) by some authors, but to be normal by others (Brambilla et al. 1995; Cameron et al. 1987; Stein and Uhde 1988). Urinary free cortisol in PDA patients was found to be normal (Uhde et al. 1988), elevated (Bandelow et al. 1997) or elevated only in patients with complicated PDA (Lopez et al. 1990) when compared with healthy controls.

Baseline ACTH concentration in plasma was found to be increased in patients compared to controls (Brambilla et al. 1992). HPA axis stimulation tests showed significantly lower ACTH responses to corticotropin-releasing hormone (CRH) in patients compared to normal control subjects in three studies (Brambilla et al. 1992; Holsboer et al. 1987; Roy-Byrne et al. 1986) and normal responses in one (Rapaport et al. 1989). Cortisol release after CRH was found to be lower in two (Brambilla et al. 1992; Roy-Byrne et al. 1986) and normal in two other studies (Holsboer et al. 1987; Rapaport et al. 1989).

#### *HPA axis response during panic attacks*

Cameron et al.(1987) measured cortisol during spontaneously occurring panic attacks while patients stayed at bedrest with an indwelling venous catheter for sampling of blood. They found non-significantly elevated plasma cortisol levels during attacks.

During naturally occurring panic attacks, a significantly increased salivary cortisol secretion could be shown in PDA patients compared to values of the same individuals obtained at comparable daytime on panic-free days (Bandelow et al. 2000). The salivary method used in this study turned out to be a useful non-invasive method to measure HPA function in anxiety disorders, which was often used in subsequent research.

During exposure to feared situations, PDA patients did not show increased levels of concentrations of cortisol and ACTH (Siegmund et al. 2011). In order to investigate cortisol levels during panic attacks, panic provocation tests have been performed. In most studies, patients who panicked during lactate infusion did not show elevations in ACTH or cortisol (Carr et al. 1986; Den Boer et al. 1989; Gorman et al. 1989; Levin et al. 1987; Ströhle et al. 1998b; Targum 1992). In a study by Liebowitz et al. (1985), only patients who rapidly developed panic attacks after lactate infusion had marginally higher cortisol levels than controls. In contrast to these findings, Hollander et al. (1989) found that cortisol levels fell significantly during lactate-induced panic in patients and controls. Interestingly, patients who panicked after lactate had higher plasma cortisol levels before the infusion than controls (Coplan et al. 1998).

Inhalation of carbon dioxide did not induce a significant increase in plasma or salivary cortisol in panickers (Gorman et al. 1989; van Duinen et al. 2004). However, subsequent studies suggested that 35% CO<sub>2</sub> significantly increases plasma levels of ACTH and cortisol in PDA patients (van Duinen et al. 2007) and of cortisol in healthy subjects (Argyropoulos et al. 2002). Nevertheless, in PDA patients, no specific association emerged between the 35% CO<sub>2</sub>-induced panic attacks and the HPA-axis' activation observed after this challenge (van Duinen et al. 2007). Patients reporting yohimbine-induced panic attacks had significantly larger increases in plasma cortisol than healthy subjects (Charney et al. 1987). M-chlorophenylpiperazine (mCPP) or oral caffeine increased plasma cortisol in both patients and controls (Charney et al. 1985; Klein et al. 1991). However, a placebo-controlled study suggested that the significant increases in plasma cortisol, ACTH and dehydroepiandrosterone sulfate observed after oral caffeine (400 mg) administration in PDA patients are not associated with the occurrence or not of a panic attack at post-challenge (Masdrakis et al. 2015). Pentagastrin (CCK-4) induced panic attacks were associated with a pronounced rise of plasma cortisol levels (Abelson et al. 2007).

#### *HPA axis response to treatment*

Some studies investigated the effect of treatment on the HPA axis in patients with PDA. Nocturnal urinary cortisol excretion did not change during treatment with paroxetine vs. placebo combined with relaxation training or aerobic exercise (Wedekind et al. 2008). On the contrary, exercise training was associated with lowered salivary cortisol levels in PDA patients (Plag et al. 2014).

### *HPA axis suppression tests*

Findings with the dexamethasone suppression test were summarized by Ising et al. (2012). Most studies found a normal reaction in the dexamethasone suppression test in PDA patients, e.g. Cameron & Nesse (1988), while cortisol non-suppression after dexamethasone was found in at least some patients in some other investigations (Avery et al. 1985; Erhardt et al. 2006; Petrowski et al. 2013). Results of studies employing the CRH stimulation test in PDA were heterogeneous. While two studies suggest an abnormal CRH response pattern in terms of a blunted ACTH response and a reduced ACTH/cortisol ratio, three studies were negative or showed inconsistent findings (Ising et al. 2012). Also, combined dexamethasone suppression/CRH tests supported the assumption of an impaired HPA axis regulation in PDA (Ising et al. 2012). Demiralay et al. (2012) found a blunted response of ACTH release following CCK-4 injection only after hydrocortisone pre-treatment.

### *HPA axis and neurotrophic factors*

According to a review, early stressful life events may provoke alterations of the stress response and the HPA axis, which can endure until adulthood (Faravelli et al. 2012). Glucocorticoids suppress brain-derived neurotrophic factors (BDNF) at messenger ribonucleic acid (mRNA) and protein level. Activated glucocorticoid and mineralocorticoid receptors repress the transcription activity of the BDNF promoter site. Neurogenesis in the human brain is in fact most prominent in the dentate gyrus of the hippocampus. Hypercortisolism caused by prolonged stress can suppress this neuroplasticity process. Acute stress, however, activates BDNF, stimulates neuroplasticity and hence improves learning and memory. Therefore, under chronic stress conditions such as in PDA, an increasing loss of neural plasticity may emerge and consequently the ability to appropriate coping (Bandelow and Wedekind 2006). The role of neurotrophic factors is reviewed in the next chapter (Neurotrophic factors, page 33).

## **GAD**

### *Basal levels and HPA axis response to stressors*

It is uncertain, as yet, whether untreated GAD is associated with abnormally increased cortisol levels. Thus, some studies suggest that GAD patients and controls demonstrate similar baseline cortisol levels and cortisol responses to challenge tests. More precisely, baseline



urinary free cortisol levels between patients with “chronic moderate-to-severe anxiety” and normal controls did not differ significantly (Rosenbaum et al. 1983). Twenty GAD male adolescents and normal controls displayed similar cortisol plasma levels after a stressful test, but anxious subjects had demonstrated greater pre-stress ACTH concentrations (Gerra et al. 2000). In an extensive study with 1427 anxious patients and normal controls, GAD patients demonstrated significantly greater cortisol awakening response than controls, only when also suffering major depressive disorder (MDD) (Vreeburg et al. 2010). Among 4256 Vietnam-era veterans, those suffering from GAD and normal controls showed similar cortisol and dehydroepiandrosterone sulfate (DHEAS) plasma levels and cortisol/DHEAS ratio (Phillips et al. 2011). Corresponding to younger subjects, baseline cortisol levels of 201 elderly subjects with at least one anxiety disorder (including GAD and phobias) were comparable to those of normal controls. However, under stress, males showed a slower decline rate of post-stress cortisol increases compared to controls, while clinical severity was associated with larger post-stress cortisol increases and lower recovery capacity in females (Chaudieu et al. 2008). Administration of 7.5% carbon dioxide did not significantly change salivary cortisol levels in medication-free GAD patients (Seddon et al. 2011). Finally, 7- to 11-year-old children with GAD did not differ from controls concerning pre-sleep salivary cortisol, despite the presence of sleep disturbances (Alfano et al. 2013).

*On the contrary, other studies report abnormal – either increased or decreased – HPA axis activity in GAD.* Thus, in elderly GAD patients, compared to non-anxious controls, cortisol levels were overall significantly more elevated, were higher during morning hours and were positively associated with GAD symptoms (Mantella et al. 2008). Moreover, not only untreated but also SNRI-treated GAD patients demonstrated significantly higher cortisol levels compared to normal controls (Hood et al. 2011). A recent development is the analysis of hair cortisol concentrations, which reflect the long-term cortisol levels independently of the acute HPA axis responses in the laboratory context. GAD patients demonstrate up to 50-60% lower hair cortisol concentrations compared to healthy controls (Staufenbiel et al. 2013; Steudte et al. 2011). These results are in accordance with the notion that chronic anxiety – an essential clinical feature of GAD – may result in down-regulation of HPA axis activity. Thus, older adults ( $\geq 65$  year-old) suffering from long-lasting anxiety disorders demonstrated a lower cortisol awakening response than normal controls. This association was most prominent in GAD patients, however, irrespectively of the duration of illness (Hek et al. 2013). Likewise, chronic anxiety may finally exhaust the capacity for increase in serotonin transporter due to

the chronically elevated plasma cortisol levels, e.g. GAD patients could not increase serotonin uptake in their lymphocytes after cortisol administration (Tafet et al. 2001).

#### *HPA axis suppression tests*

Non-suppression in the dexamethasone suppression test (DST) in GAD patients (up to 27%) is comparable to that of MDD outpatients, but seems to have little value in distinguishing between GAD and other disorders, including PDA, MDD and agoraphobia (Avery et al. 1985; Okasha et al. 1994; Schittecatte et al. 1995; Schweizer et al. 1986; Tiller et al. 1988).

#### *HPA axis response to treatment*

Some studies report that successful psychological or pharmacological treatment of GAD is associated with post-treatment cortisol level reductions. Thus, after successful CBT treatment for GAD, significant decreases in both anxiety symptoms and (the elevated at baseline) plasma cortisol levels were observed (Tafet et al. 2005). GAD patients over 60 years of age displayed greater reductions in both peak and total salivary cortisol after escitalopram treatment, compared to placebo-treated patients (Lenze et al. 2011). Furthermore, cortisol reductions were positively associated with improvements in anxiety, although this was limited to subjects with elevated (above the median) baseline cortisol levels. Of note, genetic variability at the serotonin transporter promoter predicted these cortisol changes. Furthermore, in the escitalopram (but not in the placebo) treatment group, salivary cortisol changes were significantly associated with changes in immediate and delayed memory tasks, suggesting that targeting HPA axis dysfunction may improve memory in older GAD patients (Lenze et al, 2012). Tiller et al. (1988) reported that all GAD patients who were DST non-suppressors at pre-treatment were suppressors after successful behavioural treatment. Finally, refocusing GAD patients' attention (and thus distracting them from their anxious thoughts) seems to reduce cortisol levels (Rosnick et al (2013).

However, other studies report no association between a positive treatment outcome and post-treatment changes in cortisol levels, or no change of cortisol levels at all. Thus, effective treatment of GAD either with buspirone (Cohn et al. 1986) or with alprazolam (Klein et al. 1995) did not significantly alter cortisol levels. Intravenous administration of diazepam in eight GAD patients was associated with post-challenge reductions in cortisol (dose-dependently) and ACTH (dose-independently) (Roy-Byrne et al. 1991). There was no interaction with diagnosis for any of these endocrine measures, indicating no differential effects of diazepam on ACTH or cortisol in the GAD and control groups. Subsequently, in a larger study in GAD patients and healthy controls, diazepam reduced plasma cortisol levels

both when acutely administered at baseline and during chronic treatment and this effect was most apparent in the elderly (60–79 years) compared with the young adults (19–35 years) (Pomara et al. 2005). However, this effect was not associated with the presence of GAD.

## **SAD**

The HPA axis is an important stress system concerning social interaction. Primates with higher baseline HPA axis activity and greater reactivity to stressful stimuli demonstrate increased social avoidances (Kalin et al. 1998; Sapolsky and Plotsky 1990). Consequently, research concerning the pathophysiology of SAD has focused on the potential role of cortisol in regulating cognitive processes and behavioural responses (e.g. avoidances) to social stressors (de Kloet et al. 1999; Elnazer and Baldwin 2014; Roelofs et al. 2009; Sapolsky 1990; van Peer et al. 2010).

### *Basal levels and HPA axis response to stressors*

Some studies suggest that baseline cortisol levels or cortisol responses after pharmacological or psychological challenges are similar between SAD patients and controls. Thus, no evidence of HPA axis hyperactivity in SAD patients compared to healthy controls was observed, as this is reflected in urinary free cortisol levels or in the free cortisol/creatinine ratio (Potts et al. 1991), as well as in the 24-hour excretion of urinary free cortisol and in post-dexamethasone cortisol levels (Uhde et al. 1994). Additionally, diurnal saliva cortisol levels and cortisol increases observed both before attending school and before a Trier Social Stress Test were similar between 27 adolescent girls with SAD and healthy controls (Martel et al. 1999). Moreover, SAD patients, compared to controls, demonstrated significantly greater ACTH and cortisol responses to stress (Young et al. 2004) and a significantly greater cortisol awakening response (Vreeburg et al. 2010), only when suffering major depression as well. Intravenous administration of CCK-4 in SAD or OCD patients, or normal controls failed to find any significant between-groups differences concerning post-challenge ACTH, cortisol, growth hormone and prolactin responses (Katzman et al. 2004). Intravenous administration of citalopram in SAD patients and healthy controls resulted in significantly greater increases in cortisol and prolactin plasma levels compared to placebo administration, which were yet similar between the two groups (Shlik et al. 2002). Although a rapid intravenous meta-chlorophenylpiperazine (m-CPP) challenge resulted in significantly greater rate of panic attacks in PDA patients (85%) compared to generalized SAD patients (14%) and healthy

controls (0%), yet post-challenge changes in cortisol levels were comparable between these groups (Van Veen et al. 2007).

In SAD patients evaluated at baseline and after dexamethasone, no differences were found concerning cortisol awakening response, post-dexamethasone and other cortisol measurements, in contrast to the observed elevations in diurnal and post-dexamethasone levels of salivary alpha-amylase, a marker of autonomic nervous system function (van Veen et al. 2008). Subsequently, SAD patients successfully treated with a SSRI underwent either a tryptophan depletion challenge or a placebo-test, combined with a public speaking-challenge. The tryptophan depletion group showed a significant larger salivary  $\alpha$ -amylase response compared to the placebo-group, yet the two groups demonstrated similar salivary cortisol responses (van Veen et al. 2009). Accordingly, SAD patients who underwent an electrical stimulation test demonstrated significantly greater baseline and post-challenge salivary  $\alpha$ -amylase levels compared to controls. Concerning salivary cortisol levels, neither within-subject nor group differences were observed (Tamura et al. 2013). These findings have led some researchers to suggest that pathological vulnerability of the autonomic nervous system – and not of the HPA axis – may underlie SAD psychopathology (Tamura et al. 2013; van Veen et al. 2009; van Veen et al. 2008). However, both salivary cortisol and  $\alpha$ -amylase levels were similar between SAD children (aged 8–12 years) and healthy controls after undergoing the Trier Social Stress Test for Children, although the former demonstrated significantly higher reactivity compared to the latter (Kramer et al. 2012).

On the contrary, other studies suggest that SAD patients differ significantly from controls concerning baseline cortisol levels and/or cortisol responses to pharmacological or psychological challenges. Thus, in SAD patients, administration of fenfluramine (Tancer et al. 1994b) or m-CPP (Hollander et al. 1998) resulted in significantly greater cortisol responses compared to controls. Furlan et al (2001) reported different dichotomies in magnitude and in distribution of cortisol responses to a speech-stressor between SAD patients and normal controls. Thus, seven patients and 14 controls demonstrated post-challenge cortisol increases (90% and 50% respectively), while in the remaining 11 patients and three controls, cortisol decreased. Of note, both patients' groups were significantly more anxious at post-challenge compared to controls. On the contrary, SAD patients and controls showed similar cortisol responses to a physical exercise-challenge, suggesting that distinct biological processes underlie responses to different stressors in SAD (Furlan et al. 2001). Patients with SAD, compared to healthy controls, had a significantly larger cortisol response when performing an

arithmetic/working memory task in front of an audience (Condren et al. 2002). Baseline ACTH and cortisol, as well as post-challenge ACTH responses were all similar between the two groups. Exaggerated cortisol response to a speech-stressor was suggested to be a potential neurobiological marker for pre-pubertal SAD children (van West et al. 2008). Moreover, an elevated afternoon salivary cortisol level at the age of 4.5 years was one of the four risk factors (the rest being female gender, early exposure to maternal stress and early manifestation of behavioural inhibition) mediating the association between chronic high inhibition in school age and SAD occurrence during adolescence (Essex et al. 2010). Additionally, in adolescents, a higher baseline cortisol awakening response significantly predicted increased first onsets mainly of SAD (among other anxiety disorders) over a six-year follow-up (Adam et al. 2014). Finally, recent data suggest that 8–12-year-old children with an anxiety disorder (including SAD, GAD, specific phobia and SePAD) demonstrate psychophysiological characteristics resembling those of chronic stress, i.e. a baseline pattern comprising reduced HPA axis functioning and elevated sympathetic and lowered parasympathetic activity compared to controls (Dieleman et al. 2015).

Increased cortisol stress-responsiveness may be linked to increased social avoidance behaviours in SAD patients. Indeed, SAD patients showed larger cortisol responses to a social stressor, compared to healthy controls. Most crucially, cortisol responses correlated positively to avoidance behaviours displayed during the social-stressor and, furthermore, predicted them irrespectively of blood pressure and anxiety (Roelofs et al. 2009). The authors speculate that some studies failed to find an increased HPA axis response to social stressors in SAD patients due to protocol violations – e.g. manipulations that reduce a patient's experimentally-induced stress in order to avoid drop-out of the patient – which might critically reduce their cortisol responses.

The potential role of cortisol in threat processing in SAD remains unclear. Event-related potential analysis indicated that in SAD patients, cortisol administration prior to a social stress-related reaction time task increases early processing of social stimuli (particularly angry faces) during avoidance (van Peer et al. 2009). A subsequent event-related potential study suggested a highly specific effect of cortisol on early motivated attention to social threat in SAD (van Peer et al. 2010).

#### *HPA axis response to treatment*

Clinical improvement after fluvoxamine treatment in SAD patients was *not* associated with baseline and post-treatment plasma cortisol responses to a speech-test (DeVane et al. 1999).

### *Glucocorticoids in the treatment of SAD*

Elevated glucocorticoid levels might inhibit the retrieval of fear-related memories and, thereby, reduce phobic fear. Thus, in SAD patients, cortisone administered orally one hour before a social stressor significantly reduced social fear (but not general anxiety) during the anticipation, exposure and recovery phase of the stressor. Moreover, the stress-induced release of cortisol in placebo-treated subjects correlated negatively with fear ratings, suggesting that endogenously released cortisol in a phobic context buffers fear symptoms (Soravia et al. 2006).

### **Specific Phobia**

#### *Basal levels and HPA axis response to stressors*

Most related studies suggest that specific phobia is characterized by exaggerated cortisol increases during exposure to phobic stimuli. Thus, in patients with specific phobia, exposure to phobic slides elicited larger cortisol excretion (as well as greater distress and skin-conductance responses), compared to neutral exposures (Fredrikson et al. 1985). Likewise, in women with animal phobias, cortisol levels (as well as levels of epinephrine, norepinephrine, growth hormone and insulin) significantly rose during *in vivo* exposure sessions, together with increases in anxiety, blood pressure and pulse (Nesse et al. 1985). Moreover, in two patients who underwent exposure therapy for height phobia, increased cortisol responses remained over the course of treatment despite behavioural and subjective improvements (“desynchrony”) (Abelson and Curtis 1989). Subjects with driving phobia, compared to healthy controls, had significantly greater cortisol increases during driving and its anticipation one hour before driving. Cortisol levels were similar between the two groups on a non-driving day and on morning awakening (Alpers et al. 2003). Pregnant women suffering blood-injection phobia, compared to healthy pregnant women, had a higher output of cortisol, although both groups demonstrated similar diurnal cortisol rhythms (Lilliecreutz et al. 2011).

Of note, van Duinen et al. (2010) reported that – although during exposure to phobic stimuli spider phobic patients demonstrated significantly stronger fear reaction compared to controls – yet cortisol levels were similar between both groups, suggesting thus a “desynchrony” in patients’ response systems.

### *HPA axis response to treatment*

In army recruits with protective mask phobia, exaggerated salivary cortisol secretion was observed at both baseline and post-treatment, as well as in the morning. After successful two-day intensive CBT, significant reductions in cortisol levels were observed (Brand et al. 2011). Of note, it has been suggested that phobic patients may not respond uniformly regarding HPA axis function when exposed to phobic stimuli and that this should be taken into consideration when tailoring individualised psychotherapeutic interventions. Hence, only two-thirds of women with spider phobia showed increased cortisol responses when exposed to spider photographs, while the rest, defined as “low-responsive”, showed lower cortisol responses compared to “medium-to-high responsive” non-phobic individuals (Knopf and Possel 2009).

### *Glucocorticoids in the treatment of specific phobia*

Glucocorticoid treatment seems to acutely reduce symptoms of specific phobia and might have a prolonged effect concerning fear extinction, especially in combination with exposure therapy (de Quervain and Margraf 2008; Soravia et al. 2006). Thus, in subjects with spider phobia, repeated oral administration of cortisone (25 mg) one hour before exposure to spider photographs reduced phobic (but not general) anxiety significantly more than placebo, and this effect was maintained for two days (Soravia et al. 2006). Additionally, patients fearing heights who underwent a three-session virtual-reality exposure therapy after receiving cortisol (20 mg) one hour before each session, demonstrated significant fear reduction, as well as reductions in acute anxiety and in skin conductance during exposures to phobic stimuli (de Quervain et al. 2011).

## **OCD**

### *Basal levels and HPA axis response to stressors*

Research data suggest abnormal HPA axis function at baseline and after stress in OCD patients. More precisely, children and youths with OCD displayed higher early-morning cortisol values, compared to healthy controls. Moreover, the cortisol levels in the OCD group diminished in response to a psychological stressor, while a positive response was observed in the reference group (Gustafsson et al. 2008). In adults with OCD, similar diurnal secretion patterns were found when compared to healthy controls; however, an overall increase in HPA axis activity was found in OCD patients (Kluge et al. 2007). Exposure with response

prevention was used as a stressor in patients with OCD. Despite considerable psychological stress, no increase of salivary cortisol was observed (Kellner et al. 2012).

## **PTSD**

Some studies have found lower cortisol excretion in PTSD patients. According to a review by Yehuda (2005), most studies demonstrate alterations consistent with an enhanced negative feedback inhibition of cortisol on the pituitary, an overall hyper-reactivity of other target tissues (adrenal gland, hypothalamus), or both in PTSD. However, findings of low cortisol and increased reactivity of the pituitary in PTSD are also consistent with reduced adrenal output. The possible clinical applications of HPA biomarkers have been reviewed by Lehrner & Yehuda (2014).

### *Basal levels*

Low urinary cortisol excretion was found in combat veterans with PTSD as compared to controls (Yehuda et al. 1990). Holocaust survivors with PTSD showed significantly lower mean urinary cortisol excretion than subjects without PTSD (Yehuda et al. 1995). In a small study, patients with PTSD were compared to patients with PDA and healthy controls. PTSD patients had lower cortisol and marginally reduced cortisol volatility compared to patients with panic disorder (Marshall et al. 2002). Low cortisol levels in the immediate aftermath of trauma have been found to predict the development of PTSD (Delahanty et al. 2005; Delahanty et al. 2000; Yehuda et al. 1998). A meta-analysis of 47 studies revealed that daily cortisol output was lower for PTSD patients relative to healthy controls without trauma; subjects who were exposed to trauma but did not develop PTSD did not differ from healthy controls without trauma (Morris et al. 2012).

However, in a recent study assessing hair cortisol – which reflects long-term cortisol changes –, PTSD patients and traumatized control subjects without PTSD exhibited lower hair cortisol than non-traumatized control subjects suggesting that trauma exposure *per se*, either in the absence or presence of PTSD is a correlate of long-term lower basal cortisol levels (Steudte et al. 2013).

### *Glucocorticoids in the treatment of PTSD*

Based on the above-mentioned findings of decreased cortisol concentrations in PTSD, it has been hypothesized that glucocorticoid administration may benefit patients. Indeed, individuals who received a high dose of hydrocortisone within 6 hours of a traumatic event had a reduced



risk for the development of PTSD, compared to individuals who received placebo (Zohar et al. 2011).

In summary, although the clinical picture of anxiety disorders suggest a potential prominent role of a disturbed stress response regulation, yet there are more inconsistencies than consistencies in the relevant research findings.

In PDA, findings are inconsistent regarding baseline cortisol and ACTH levels, response to spontaneously occurring panic attacks, response to exposure to feared situations, chemically provoked panic attacks or response to the dexamethasone suppression or CRH challenge.

In GAD, findings are inconsistent regarding whether baseline cortisol levels are normal or pathologically elevated, while findings from hair cortisol analysis – a recently developed technique, which reflects the long-term cortisol levels – suggest significantly lower hair cortisol concentrations. Although dexamethasone non-suppression in GAD patients is comparable to that of MDD outpatients, yet it seems to be of little value in the differential diagnosis of GAD from other mental disorders. Most – but not all – related studies suggest that successful psychotherapy or pharmacotherapy of GAD is associated with post-treatment reductions in cortisol concentrations.

With regard to patients with SAD, some – but not all – studies suggest that they differ significantly from normal controls concerning baseline cortisol levels, and/or demonstrate exaggerated cortisol stress-responsiveness possibly linked to increased social avoidances.

Regarding specific phobia, most studies suggest inflated cortisol responses during exposure to phobic stimuli, which are yet amenable to behaviour therapy.

Overall, it seems that various pathological findings are found in HPA axis function across the anxiety disorders. Nevertheless, it is not clear, as yet, whether this reflects reality, or is due to methodological weaknesses of current research. In order to more vigorously evaluate the potential role that HPA axis function plays in the pathophysiology of anxiety disorders, a number of strategies have been previously proposed, such as achieving greater consensus on study objectives and on clinical features of patients' groups and designing meticulous methodological protocols (Baldwin et al. 2010; Elnazer and Baldwin 2014).

## *Neurotrophic factors*

Neurotrophins are proteins involved in neurogenesis. Although most of the neurons in the brain are formed prenatally, some parts of the adult brain have the ability to form new neurons from neural stem cells, a process named neurogenesis. Neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3, neurotrophin-4, and artemin.

### **Nerve Growth Factor (NGF)**

Nerve growth factor (NGF) is a neuropeptide involved in the regulation of neuron growth. On the one hand, NGF may be involved in the alert mechanism associated with homeostatic adaptations (Cirulli and Alleva 2009), on the other hand, it might modulate sympathetic neurons and therefore it keeps a key position in controlling the responsiveness of immune-competent cells (Levi-Montalcini et al. 1995). Furthermore, NGF, via the hypothalamus (Scaccianoce et al. 1993), can activate the HPA axis (Otten et al. 1979) and plays a role in adaptive responses. More importantly, there is evidence that NGF might be an autocrine/paracrine factor for the development and regulation of immune cells (Levi-Montalcini et al. 1995). NGF is produced by T and B lymphocytes (Lambiase et al. 1997), which display functional NGF receptors (Franklin et al. 1995). Furthermore, NGF promotes the proliferation and differentiation of T and B lymphocytes (Brodie and Gelfand 1992), and acts as a survival factor for memory B lymphocytes (Torcia et al. 1996).

An association between trait anxiety and a genetic variation of NGF was found in healthy volunteers (Lang et al. 2008). In soldiers making their first parachute jump, NGF was increased during and after the jump (Aloe et al. 1994).

While a reduction of NGF in depression has been consistently reported (Wiener et al. 2015), NGF has not been studied widely in patients with anxiety disorders. In one GAD study, NGF was increased after successful CBT (Jockers-Scherubl et al. 2007).

### **Brain-Derived Neurotrophic Factor (BDNF)**

BDNF is a protein that acts on neurons in the brain and the peripheral nervous system, involved in neurogenesis and in the forming of new synapses. It was assumed that BDNF is implicated in the aetiologies of depression and anxiety. Data on brain BDNF levels in anxiety disorders are controversial.

### *PDA*

The serum BDNF levels of the PDA patients with poor response to CBT were significantly lower than those of the patients with good response (Kobayashi et al. 2005). Moreover, BDNF serum levels increased after 30 minutes of aerobic exercise in subjects with panic but not in healthy controls (Ströhle et al. 2010).

### *GAD*

In a treatment study with GAD patients, no significant association was found between baseline plasma BDNF levels and GAD severity. Patients who received the SNRI duloxetine had a significantly greater mean increase in plasma BDNF level compared with patients who received placebo (Ball et al. 2013). In a sample of 393 patients with panic disorder, agoraphobia, GAD or SAD, no differences in BDNF levels were found when compared to 382 healthy controls (Molendijk et al. 2012).

A small study comparing patients with GAD or major depressive disorder to healthy subjects showed doubled levels of BDNF and artemin, a glial cell-line derived neurotrophic factor family member, in GAD patients compared to normal controls, while depressed patients showed a reduction (Pallanti et al. 2014).

In summary, neurotrophic factors seem to play a different role mood disorders and anxiety. While brain atrophy and growth factor reduction have been observed in mood disorders the opposite has been demonstrated in anxiety disorders. One hypothesis could be that the increase of neurotrophic factors and inflammatory factors observed in anxiety disorders are related to brain volume increase observed in brain areas such as the dorsal midbrain by some studies on anxiety disorders (Fujiwara et al. 2011; Uchida et al. 2008) (see also Chapter neuroimaging, Part I (Bandelow et al. submitted)).

### *Immunological markers*

Neurobiological research on anxiety disorders has shown the possible relevance of neuroplasticity and inflammation processes in the pathophysiology of these disorders. The high rate of comorbidity between anxiety disorders and several inflammatory medical conditions has been interpreted as the result of specific inflammatory pathways. Anxiety has been linked to cardiovascular risk factors and diseases such as atherosclerosis (Seldenrijk et al. 2010), metabolic syndrome (Carroll et al. 2009), and coronary heart disease (Roest et al.

2010), which are also associated with low-grade systemic inflammation (Libby 2002). While depressive disorders, which are highly comorbid with anxiety disorders, have repeatedly been associated with the immune system (Kim et al. 2007; Myint and Kim 2014), only a few studies have investigated the relationship between anxiety disorders and inflammation (Vogelzangs et al. 2013). These have suggested that certain inflammatory markers are elevated in anxiety disorders (Weik et al. 2008).

### **The immune system**

The immune system is divided into the *innate* and the *acquired* immune system. The latter again is divided into the *cellular* and the *humoral* immune system. The humoral system is based on antibodies, while the cellular immune system involves the phagocytes, cytotoxic T-lymphocytes, and cytokines. Lymphocytes are white blood cells in the lymph that include thymus cells (*T cells*), which can produce enzymes that destroy pathogenic cells, bone marrow cells (*B cells*), which produce antibodies for the humoral immune system to fight bacteria and viruses, and natural killer cells, which defend the host from tumor cells and virus infections. Inflammatory responses are characterized by a complex interaction between pro- and anti-inflammatory *cytokines* (Pavlov and Tracey 2005). Cytokines are small proteins, including the *interleukins* (ILs) such IL1, -2, -6, -10, -18 and others, *tumor necrosis factors* (TNFs) and *interferons* (IFNs) such as IFN  $\alpha$ ,  $\beta$  and  $\gamma$ . Interferons are released by cells that have been infected by a virus, and are used as drugs (e.g.  $\alpha$ -interferon for the treatment of hepatitis C or cancer,  $\beta$ -interferon for multiple sclerosis or interleukin 2 for cancer). Interferons also activate natural killer cells.

Epinephrine and norepinephrine modulate the release of cytokines and inflammation through  $\alpha$ - and  $\beta$ -adrenoceptors on immune cells (Hasko and Szabo 1998). Results of *in vitro* and *in vivo* studies have suggested that norepinephrine enhances TNF production (Bertini et al. 1993; Spengler et al. 1994). TNF is an early cytokine mediator of local inflammatory response that causes inflammation and secondary tissue damage when released in excess (Tracey 2002). Both catecholamines have been reported to stimulate IL-6 release by immune cells and other peripheral cells (Chrousos 2000). Norepinephrine augments macrophage phagocytosis and tumoricidal activity (Koff and Dunegan 1985). In contrast, acetylcholine dose-dependently inhibit the release of TNF and other pro-inflammatory cytokines such as IL1, IL6, and IL18, from endotoxin-activated primary human macrophages (Borovikova et al. 2000). However, the production of IL10, which is an anti-inflammatory cytokine, was unaffected by

acetylcholine. Inhibition of acetylcholinesterase activity, which increases acetylcholine levels in the central nervous system, resulted in the suppression of the immune response, indicating that acetylcholine has an immunoinhibitory role in the brain (Pavlov et al. 2009). When stressful situations are prolonged, adrenergic agents can increase and acetylcholine can decrease, due to continuous sympathetic activation and the lack of parasympathetic counteractivation. Therefore, pro-inflammatory cytokines such as TNF, IL1, and IL6 can increase in prolonged stressful situations, such as anxiety disorders.

#### *The autonomic nervous system and the immune system*

Although stress initially activates both the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis, the role of the autonomic nervous system and its interactions with stress and the immune system has received much less attention than the HPA axis (Elenkov et al. 2000). Stress-induced interactions between nervous, endocrine and immune systems are depicted in Figure 2.

Insert Figure 2

Mental arithmetic and public speaking tasks applied as brief laboratory stressors induce increases in natural killer cell activity (Breznitz et al. 1998). These increases were potentiated in individuals who were cardiovascularly more reactive to stress (Cacioppo et al. 1995). In other words, individuals who showed the greatest sympathetic nervous system and endocrine response to brief psychological stressors, also showed increased immune system alterations. Thus, the effect of stress on the neuroendocrine system and the mechanism by which that effect influences the immune system has become a subject of interest in recent years (Larson et al. 2001).

### **Cellular Immunity**

#### *PDA*

In PDA patients, peripheral lymphocyte subsets did not differ initially from control subjects. However, after three months of treatment with the SSRI paroxetine, the percentages of some lymphocyte subsets were significantly increased, while others were decreased (Kim et al. 2004). This finding suggests that pharmacological treatment may affect immune function in panic disorder patients. In a study by Schleifer et al. (2002), drug-free patients with PDA

showed decreased percentages and total circulating CD19+ B lymphocytes, but no differences in other lymphocyte measures. Natural killer cell activity did not differ between PDA patients and healthy control subjects in this study.

### *GAD*

In a study by Wingo and Gibson (2015), anxiety as a symptom of GAD was associated with blood gene expression profiles in 336 community participants (157 anxious subjects and 179 controls). Findings did not show a significant differential expression in females, but 631 genes were differentially expressed between anxious male and healthy controls. Gene set enrichment analysis revealed that genes with altered expression levels in anxious men were involved in response of various immune cells (B-cells, myeloid dendritic cells and monocytes) to vaccination and to acute viral and bacterial infection (peripheral blood mononuclear cells). In addition, this analysis also identified a network affecting traits of metabolic syndrome. These results suggest potential molecular pathways that can explain the negative effects of GAD on physical health that are observed in epidemiological studies. Remarkably, even mild anxiety, which most of the study participants had, was associated with observable changes in immune-related gene expression levels.

### *OCD*

In OCD, circulating natural killer cells were either increased, decreased or not changed compared to controls. In one study, circulating natural killer cells were elevated predominantly in males which persisted after 12 weeks of SSRI treatment, possibly reflecting either characteristic of the illness, or a lack of true remission (Ravindran et al. 1999). Another study found that patients with childhood onset of OCD had significantly more natural killer cells than patients with late onset OCD (Denys et al. 2004). A subsequent study reported that the percentage and absolute numbers of natural killer cells measured as CD56 lymphocyte subpopulations, were unchanged (Marazziti et al. 1999). Patients with first-degree relatives with OCD also had significant lower natural killer cell activity compared to patients who had no relative with OCD (Denys et al. 2004). In a study by Marazziti et al. (1999), OCD patients had increased CD8+ T cells, both in terms of percent values and absolute number, and decreased CD4+ T cells. The CD3+, CD19+, and CD56+ lymphocyte subpopulations were unchanged.

## *Cytokines*

### *PDA*

Patient with PDA had reduced cell-mediated functions compared to healthy controls before pharmacological treatment. After treatment, no significant differences were seen (Koh and Lee 2004). One study showed increased levels of 18 cytokines in subjects with PDA and PTSD, leading the authors to suggest that a generalized inflammatory state may be present in these diseases (Hoge et al. 2009). However, small studies on cytokines in PDA showed non-significant elevations of TNF- $\alpha$ , IL1- $\alpha$ , IL2, and IL3 but a significant increase of IL1  $\beta$  (Brambilla et al. 1994; Rapaport and Stein 1994; Weizman et al. 1999). In a study conducted on PDA patients and healthy controls, plasma concentrations of TNF- $\alpha$ , IFN- $\gamma$ , IL1 $\beta$ , IL2, IL6, and IL12 were measured. Decreased levels of IFN- $\gamma$  and IL12 were observed, which suggested a correlation between levels of IFN- $\gamma$  and anxiety-like behaviour, as seen in animal models (Tukel et al. 2012).

### *GAD*

In GAD, C-reactive protein was increased in some studies (Bankier et al. 2008; Copeland et al. 2012). A pilot study measured peripheral levels of relevant cytokines (alpha-MSH, IL2 and IL10) in small cohorts of GAD and MDD patients and compared them to healthy controls. They found increases in plasma concentrations of IL10 and alpha-MSH, but no significant variations in IL2 (Tofani et al. 2015). One study conducted on patients with GAD and PDA measured cell-mediated immune functions through the lymphocyte proliferative response to phytohemagglutinin (PHA), interleukin-2 (IL2) production and natural killer cell activity. This study suggested a reduction in this function when compared to healthy controls (Koh and Lee 1998).

### *SAD*

Among individuals with anxiety disorders, those with SAD, females in particular, had lower levels of C-reactive protein (CRP) and IL6. The highest CRP levels were found in those with an older age at anxiety disorder onset (Vogelzangs et al., 2013). CRP is an acute-phase protein produced in the liver that increases stimulated by IL6, which is in turn secreted by macrophages and T cells.

### *OCD*

Different methodologies, including *ex vivo* production and peripheral blood or CSF measurements via a variety of techniques, make comparisons difficult. Several studies

(Fluitman et al. 2010; Mittleman et al. 1997) have shown that cytokine levels may depend on factors such as age, and the content of obsessions. For example, a study by Fluitman et al. (2010) showed that norepinephrine levels increased while lipopolysaccharide-stimulated TNF- $\alpha$  and IL6 production by peripheral leucocytes decreased during exposure to disgust-related objects in OCD patients, but not in healthy controls. These data suggest that symptom provocation in OCD patients with contamination fear is accompanied by alterations in the immune and neuroendocrine systems, but does not affect cortisol levels.

In OCD, several studies demonstrated diminished production of TNF- $\alpha$  (Brambilla et al. 1997; Denys et al. 2004; Fluitman et al. 2010). One of the first studies in the field (Brambilla et al. (1997) showed lower plasma concentrations of IL1 $\beta$  and TNF- $\alpha$  in OCD patients compared to controls, which has been related to hyperactivity of the noradrenergic system and of the HPA axis. In a study by Denys et al. (2004), the *ex vivo* production of TNF- $\alpha$  in whole blood cultures was significantly decreased in medication-free patients with OCD compared to controls. The same study showed reduced natural killer cells activity. The reduction in both TNF- $\alpha$  and natural killer cells activity suggests a potential role of altered immune function in the pathophysiology of OCD. Other studies have revealed normal cytokine production in OCD patients (Weizman et al. 1996). On the other hand, the possible involvement of the immune system in certain subtypes of OCD is supported by the relationship between the severity of the disorder and the IL6/IL6 receptor levels (Maes et al. 1994). However, childhood OCD appears to differ from that occurring at other ages, as increased CSF levels of cell-mediated cytokines have been reported in children with OCD, when compared to children with schizophrenia or attention deficit hyperactivity disorder (Mittleman et al. 1997). Hounie et al. (2008) reported a genetic association between the -308 G/A and -238 G/A TNF- $\alpha$  polymorphisms and OCD in a Brazilian sample.

### *PTSD*

Cytokines levels appear to be constantly elevated in PTSD. Some studies have reported higher plasma IL6 and TNF (Gill et al. 2008; von Kanel et al. 2007), and CSF IL6 levels (Baker et al. 2001) among PTSD. Higher levels of IL6 are linked to PTSD vulnerability following trauma (Gill et al. 2009; Pervanidou et al. 2007; Sutherland et al. 2003). Higher levels of stimulated TNF and IL6 were reported in PTSD patients. In a study by Rohleder et al. (2004), LPS-stimulated production of IL6, but not TNF- $\alpha$ , was markedly increased in patients. Spivak et al. (1997) showed that serum IL1 $\beta$  levels (but not sIL-2R) were significantly higher in PTSD patients than in controls. As these levels correlated significantly with the duration of PTSD



symptoms, it was proposed that desensitization of the HPA axis in chronic PTSD patients counteracted the stimulatory effect of IL1 $\beta$  on cortisol secretion. Another study showed that levels of TNF- $\alpha$  and of IL1 $\beta$  were higher in patients than in controls, while C-reactive protein (CRP), IL4, and IL10 were not significantly different (von Kanel et al. 2007). One study found higher IL1  $\beta$  and lower IL2R levels in PTSD patients than in control subjects (Tucker et al. 2004). In all subjects, TNF- $\alpha$  was correlated with PTSD severity. IL4 correlated with total hyperarousal symptoms, and PTSD total symptom score, after controlling for systolic blood pressure and smoking status. PTSD patients showed a low-grade systemic proinflammatory state that was related to disease severity suggesting one mechanism by which PTSD could contribute to atherosclerotic disease. A study by Miller et al. (2001) reported a positive relationship between posttraumatic psychological disturbances and serum levels of receptors to interleukin 6 (sIL6r) and CRP, which provides the basis for further research on the effects of psychological disturbance on physical recovery after injury.

## **Humoral Immunity**

### *PDA*

Mannan-binding lectin (MBL) and mannan-binding lectin-associated serine protease-2 (MASP-2) represent important arms of the innate immune system, and different deficiencies may result in infections or autoimmune diseases. Although PDA was associated with increased inflammatory response, infections and high comorbidity, the basis for these findings is not clear. A study by Fodager et al. (2014) investigated associations with MBL, MASP-2 or the gene MBL2 (which codes for MBL) with PDA. A large proportion (30%) of MBL deficient individuals was observed along with significantly lower levels of MBL and MASP-2 plus association with the MBL2 YA two- marker haplotype. Since MBL deficiency is highly heterogeneous and associated with both infectious and autoimmune states, more research is needed to identify which complement pathway components could be associated with PDA.

### *Antibodies*

#### **PANDAS (PANS/CANS)**

OCD is a clinically heterogeneous disorder with several possible subtypes. It has been hypothesized that one of these subtypes is associated with autoimmune disorders triggered by streptococcal infections (e.g. rheumatic fever and Sydenham's chorea) (Miguel et al. 2005). Children who develop acute OCD after a group A streptococcal (GABHS) infection were first

described by Swedo (2002), who coined the acronym PANDAS (Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococci). However, as the etiology of the syndrome remains controversial, new descriptions have been proposed, including pediatric acute-onset neuropsychiatric syndrome (PANS) and idiopathic childhood acute neuropsychiatric syndrome (CANS) (APA 2013).

Children with PANDAS showed OCD symptoms and tics, but did not have rheumatic fever or Sydenham's chorea. It has also been reported that 4% of parents and grandparents of Sydenham's chorea patients and 6.7% of the parents and grandparents of PANDAS patients developed rheumatic fever compared to 1.4% of parents and grandparents of controls. This suggests a common liability between rheumatic fever and OCD triggered by streptococcus infections (Swedo 2002). The presence of autoantibodies due to molecular mimicry mechanisms is one potential explanation for the association between OCD and rheumatic fever, following the autoimmune model for Sydenham's chorea.

Infections with group A  $\beta$ -hemolytic streptococci might result in PANDAS, viral infections might trigger the autoimmune process that leads to OCD (Allen et al. 1995; Khanna et al. 1997). Furthermore, patients with rheumatic fever show a high level of antineural antibodies against the caudate (Husby et al. 1976). They also have high levels of a monoclonal antibody called D8/D17, which reacts with a particular antigen in B lymphocytes (Zabriskie 1986). The search for the trait marker for susceptibility (Singer and Loiselle 2003) showed that this antigen is also present in patients with childhood OCD, Tourette syndrome, and chronic tic disorder (Murphy et al. 1997). This D8/D17 antibody has expanded expression in individuals with Sydenham's chorea (89%) compared with healthy children (17%). Preliminary studies of the D8/D17 antibody in individuals with PANDAS also found that 85% of children with PANDAS compared with 17% of healthy children have this antibody (Swedo et al. 1997). The exact significance of these findings and how this marker is related to the disease process is remain unclear, especially since it has been reported in patients with other neuropsychiatric disorders of childhood onset, including autism (Hollander et al. 1999; Murphy et al. 1997).

The autoimmune hypothesis has been suggested for early onset OCD and Tourette syndrome. Antineural antibodies have been studied and found in the sera of some patients with these disorders, and they are thought to cross-react with streptococcal and basal ganglia antigens (Morer et al. 2008). Positive anti-basal ganglia antibodies were found in 64% of PANDAS patients but in only 9% of controls with a documented streptococcal infection but no neuropsychiatric symptoms (Pavone et al. 2004). Immunoblotting has identified multiple

bands against the caudate supernatant fraction in PANDAS with primary tics that are different from the control group (Church et al. 2004). The presence of antibrain antibodies was reported in 42% of a group of children with OCD compared with rates between 2% and 10% in three different pediatric control (autoimmune, neurological and streptococcal) groups (Church et al. 2004). In addition, antibodies from a Sydenham's chorea patient reacted against lysoganglioside and N-acetyl-beta-D-glucosamine, a neuronal antigen that is also found on the GABHS surface (Kirvan et al. 2003). In a second study of the same group (Kirvan et al. 2006), antibodies in PANDAS reacted with the neuronal cell surface and the caudate-putamen and induced calcium-calmodulin dependent protein (CaM) kinase II activity in neuronal cells. Depletion of serum IgG abrogated CaM kinase II cell signaling and reactivity of CSF was blocked by streptococcal antigen N-acetyl-beta-D-glucosamine (GlcNAc). Antibodies against GlcNAc in PANDAS sera were inhibited by lysoganglioside GM1. Results suggest that antibodies from an infection may signal neuronal cells in some behavioural and movement disorders.

Dale et al. (2006) have identified antibodies against neuronal glycolytic enzymes (NGE) autoantigens (pyruvate kinase M1, aldolase C, neuronal-specific and non-neuronal enolase) in 20 unselected post-streptococcal patients with central nervous diseases compared to 20 controls. These enzymes are multifunctional proteins that are expressed intracellularly and on the neuronal cell surface. On the neuronal plasma membrane, NGEs are involved in energy metabolism, cell signaling and synaptic neurotransmission. GABHS also expresses glycolytic enzymes on cell surfaces that have 0–49% identity with human NGE. This suggests molecular mimicry and autoimmune cross-reactivity may be the pathogenic mechanism in post-streptococcal CNS disease. Kansy et al. (2006) identified the M1 isoform of the glycolytic enzyme pyruvate kinase (PK) as an autoimmune target in Tourette syndrome and associated disorders. Antibodies to PK reacted strongly with surface antigens of infectious strains of streptococcus, and antibodies to streptococcal M proteins reacted with PK. Moreover, immunoreactivity to PK in patients with exacerbated symptoms who had recently acquired a streptococcal infection was 7-fold higher compared to patients with exacerbated symptoms and no evidence of a streptococcal infection. These data suggest that PK can also function as an autoimmune target and that this immunoreactivity may be associated with Tourette syndrome, OCD, and associated disorders.

Further support for the autoimmune hypothesis comes from evidence of induced stereotypic movements in rats after infusion of IgG of sera from patients with PANDAS (Taylor et al.

2002). The pathogenic role of these antibodies remains unclear. Specific binding with molecules from the GABHS surface, such as lysoganglioside or glucosamine, and more neuronal glycolytic enzymes as piruvate kinase, aldolase or enolase support the notion of an autoimmune brain disease (Dale et al. 2006; Kirvan et al. 2003). However, these antibodies might not be pathogenic, but may instead result from local damage.

It should be acknowledged that some studies do not support the autoimmune hypothesis. If proved true, this hypothesis gives rise to new therapeutic approaches. In fact, some studies suggest that immuno-modulating strategies are effective in children with PANDAS (Garvey et al. 1999; Murphy and Pichichero 2002; Perlmutter et al. 1999; Snider et al. 2005). A study by Perlmutter et al. (1999) has demonstrated an improvement of obsessive–compulsive symptoms after plasmapheresis or intravenous immunoglobulin treatment. Twenty-nine children with PANDAS recruited from a nationwide search were randomized in a partially double-blind fashion (no sham apheresis) to an immunoglobulin, “immunoglobulin placebo” (saline), and plasmapheresis group. One month after treatment, the severity of obsessive-compulsive symptoms improved by 58% and 45% in the plasmapheresis and immunoglobulin groups, respectively, compared with only 3% in the saline control group. In contrast, tic scores significantly improved only after plasmapheresis treatment, but not in the immunoglobulin and the control group. Improvements in both tics and obsessive-compulsive behaviours were sustained for one year.

Even though PANDAS is by definition a paediatric disorder, patients with adult onset (after the age of 27) OCD or tic disorders related to streptococcal infections have been described. These cases support the hypothesis that streptococcal disease may result in adult-onset OCD in some patients. It is possible that GABHS infection just serves as a trigger in childhood, and that autoimmune antibodies directed against neuronal structures later maintain obsessive–compulsive symptoms without new infections. In such cases, adult OCD with childhood onset may show anti-brain antibodies without elevated ASLO titres or other signs of recent streptococcal infections. For a small proportion of OCD patients, autoimmune reactions towards neuronal structures are present, but further investigations are needed to demonstrate their etiopathogenetic relevance (Maina et al. 2009). The vast majority of OCD patients are diagnosed and treated for the first time while they are already adults; the mean time from initial symptom manifestation to seeking professional care is approximately 10 years (Maina et al. 2009).

Immunologic alterations appear to be different in pediatric and adult patients and probably reflect different pathophysiologic mechanisms, such as primary processes in the first case, and perhaps, secondary alterations in adulthood (Marazziti et al. 1999).

A study by Maina et al. (2009) showed that the proportion of subjects with tic comorbidity or positive ASLO titre ( $>200$  IU/ml) was significantly greater in OCD than in major depressive disorder patients. No other differences in antibody parameters were found. Four of 74 OCD patients (5.4%) and none of the controls were positive for anti-brain antibodies. The majority of adult OCD patients do not seem to have autoimmunity disturbances. However, a greater percentage of subjects with OCD have positive ASLO titers. For a small proportion of OCD patients, autoimmune reactions towards neuronal structures are present although further investigations are needed to demonstrate their etiopathogenetic relevance.

Two studies evaluated antineuronal antibodies or other markers of autoimmunity in samples of adult OCD patients; Black et al. (1998) found no humoral evidence of autoimmunity, but the study has certain limitations. The sample was small and heterogeneous, the severity of symptoms was not assessed at the time that blood was drawn, and an age- and gender-matched control group was not utilized. In a second study, child onset OCD was associated with higher mean ASLO titers and higher frequencies of tic disorders and tonsillitis in childhood, while no differences were found in D8/17 antibody titers or in other autoimmune parameters (Morer et al. 2006). This study suggested that OCD in adults is a heterogeneous disorder and that only child onset OCD is related to an autoimmune etiology. This topic needs further investigation, as the possible autoimmune etiopathogenesis in some OCD patients could lead to new therapeutic scenarios for adults similar to those already suggested for the children. In fact, as a significant proportion of adult OCD patients do not respond to conventional treatment strategies, the search for alternative and hypothesis-driven treatments is critical.

Early detection of these conditions through serum search of antibodies against human brain enolase, neural tissue and *Streptococcus* can provide valuable information regarding etiopathogenesis and suitable therapies (Nicolini et al. 2015). While prophylactic antibiotic therapy is marginally helpful in preventing symptom exacerbation, intravenous immunoglobulin therapy, plasmapheresis and immunosuppressive doses of prednisone may be effective treatments in select individuals (Allen et al. 1995; Nicolini et al. 2015; Swedo et al. 2001).

In conclusion, elevated levels of pro-inflammatory cytokines such as TNF, IL1, and IL6 could serve as biological markers of anxiety disorders. TNF, IL1, and IL6 trigger the activation of both the HPA axis and the sympathetic nervous system (Chrousos 1995), which could prolong the inflammatory state. The effects of these cytokines are synergistic when produced in combination (Chrousos 2000). In accordance with our current understanding of how anxiety disorders represent a state of inflammation, previous studies have attempted to investigate whether anti-inflammatory drugs have treatment effects on anxiety disorders or other psychiatric disorders deeply related to stress and anxiety. Several human and animal studies have suggested that certain anti-inflammatory drugs might play an important adjunctive role in the treatment of major depression, bipolar disorder, and OCD (Najjar et al. 2013). Although only a few studies have reported positive results for the efficacy of anti-inflammatory drug treatment on anxiety disorders (Rodriguez et al. 2010; Sayyah et al. 2011), such results do illuminate the pro-inflammatory nature of anxiety disorders. As such inflammatory conditions are considered to be triggered by an over-driven sympathetic nervous system together with an under-driven parasympathetic nervous system, treatments that increase parasympathetic tone and hence strengthen the cholinergic anti-inflammatory pathway (Pavlov 2008) could be useful in treating anxiety related disorders. This may explain why methods that increase parasympathetic tone, such as vagus nerve stimulation, may be effective in treating anxiety disorders (George et al. 2008).

### *CO<sub>2</sub> hypersensitivity*

Inhalation of air ‘enriched’ with an increased proportion of carbon dioxide (CO<sub>2</sub>) can be used to induce anxiety in non-clinical (healthy volunteers) and clinical (patients) groups, and represents a human translational model aiding development of potential new treatments for anxiety disorders. CO<sub>2</sub> inhalation has become one of the most frequently used experimental approaches to investigating panic, although studies employ variable challenge procedures, altering the CO<sub>2</sub> concentration, the duration of inhalation, the population sample, and the range of outcome measures.

Anxiety induction via CO<sub>2</sub> challenge was first performed in a small sample of patients with PDA undergoing 5% CO<sub>2</sub> inhalation, and was found to induce panic attacks (Gorman et al. 1984). This finding was confirmed in a larger sample of PDA patients, who experienced a greater incidence of panic attacks during challenge than did healthy controls or patients with other anxiety disorders (Gorman et al. 1988). Brief inhalation of air with high concentrations

of CO<sub>2</sub> (such as single vital capacity inhalations of 35% CO<sub>2</sub>) is associated with the experience of acute severe anxiety, which often includes panic attacks. A single vital capacity breath of air enriched with 35% CO<sub>2</sub> was found to induce panic and so was suggested as an approach for conducting exposure therapy in patients with PDA (Van den Hout and Griez 1984); the same group reported that patients with panic disorder were more sensitive to CO<sub>2</sub> challenge than were healthy controls (Griez et al. 1987). Findings from subsequent studies in a range of diagnostic groups indicated that panic disorder patients were more sensitive to the panicogenic effects of CO<sub>2</sub> challenge than were patients with other diagnoses (Leibold et al. 2015; Vollmer et al. 2015).

The mechanisms underlying the provocation of anxiety by CO<sub>2</sub> challenge are not fully established, although findings from animal models and human pharmacological intervention studies provide many insights (Leibold et al. 2015; Vollmer et al. 2015). Twin studies suggest an association between genetic factors and CO<sub>2</sub> hypersensitivity (Battaglia et al. 2007; Battaglia et al. 2008). Inhalation of air enriched with a high proportion (35%) of CO<sub>2</sub> may be associated with increased cortisol secretion (Argyropoulos et al. 2002; Kaye et al. 2004), although it is unclear how specific the cortisol response is to CO<sub>2</sub> challenge, rather than to other aspects of the experimental procedure (Leibold et al. 2015): most studies employing lower CO<sub>2</sub> concentrations find no increase in cortisol levels, when compared to baseline (Coplan et al. 2002; Kaye et al. 2004; Woods et al. 1988). The potential role of disturbances in respiratory physiology in panic attack induction through CO<sub>2</sub> inhalation is not fully clarified, but experimentally induced panic attacks are associated with low end-tidal CO<sub>2</sub> and high ventilation variance at baseline (Papp et al. 1997).

Serotonergic mechanisms may influence the panic response to CO<sub>2</sub> challenge. Although tryptophan depletion does not have panicogenic effects (Goddard et al. 1994), depletion can enhance the panic response to CO<sub>2</sub> inhalation (Schruers et al. 2000), and administration of the 5-HT precursor L-5-hydroxytryptophan can reduce the panic response (Schruers et al. 2002). Correlations between increases in subjective anxiety, heart rate and blood pressure in healthy volunteers following 35% CO<sub>2</sub> challenge suggest a common and presumably noradrenergic-mediated mechanism underlying CO<sub>2</sub> sensitivity (Bailey 2003). Most norepinephrine (NE) in the brain is synthesised by neurones originating in the locus coeruleus, and afferent locus coeruleus neurones project to components of the limbic system that are known to be overactive in anxiety disorders (Martin et al. 2010). Changes in CO<sub>2</sub> saturation may act upon pH or CO<sub>2</sub>-dependent chemoreceptors within the locus coeruleus and thereby increase the

release of NE, as 5% CO<sub>2</sub> increases locus coeruleus neuronal firing rate in rat brain slices (Martin et al. 2010). This CO<sub>2</sub>-induced release of NE may mediate autonomic and subjective features of anxiety through afferent projections to brain centres involved in cardiovascular control and the limbic system; and endocrine responses may be mediated by altered noradrenergic input into the paraventricular nucleus, thereby causing release of corticotrophin releasing factor (CRF) and anti-diuretic hormone, and triggering subsequent cortisol secretion.

There are limitations in an explanation of the anxiogenic effects of CO<sub>2</sub> challenge which is based solely on altered NE function. For example, autonomic arousal is not consistently observed, and the effect of 7.0–7.5% CO<sub>2</sub> on plasma cortisol is inconsistent. The attenuating effect of benzodiazepines and certain SSRIs on self-report anxiety but not on physiological markers suggest alterations in autonomic function may lie upstream of psychological anxious responding (Bailey et al. 2011a). Drugs which affect noradrenergic function have shown little effect on subjective responses to CO<sub>2</sub> (Pinkney et al. 2014). Overall, it appears that while norepinephrine may be important in mediating anxiety provoked by 35% CO<sub>2</sub> challenge, there is persisting uncertainty about the exact mechanism underlying 7.5% CO<sub>2</sub>-induced anxiety in humans.

Chemosensors within the amygdala are known to be directly linked to CO<sub>2</sub> reactivity in mice (Ziemann et al. 2009). The most well-characterized chemosensor is the acid-sensing ion channel 1 (ASIC-1a) which is a voltage-insensitive H<sup>+</sup>-gated cation channel, highly expressed in the amygdala, dentate gyrus, cortex, striatum and nucleus accumbens (Wemmie 2011). Inhalation of 2–20% CO<sub>2</sub> elicits normal mouse fear behaviour in the presence of fully functioning acid-sensing ion channels (ASIC1a), which are expressed in the amygdala, but pharmacological blockade or elimination of ASIC1a in knockout mice impairs fear responses to CO<sub>2</sub>, whereas subsequent amygdala-localised re-expression restores fear behaviour. Other potentially relevant chemosensitive structures include orexin neurones in the hypothalamus, serotonergic neurones in the medullary raphe (Wang et al. 1998), T-cell death-associated gene-8 receptors in the subfornical organ, and hypoxia-sensitive chemosensory neurones in the periaqueductal gray (Vollmer et al. 2015). Perturbations in the activities of chemosensors may not fully explain the physiological effects of changes accompanying CO<sub>2</sub> challenge and may not translate to humans, but suggest potential additional mechanisms which operate alongside CO<sub>2</sub>-provoked alterations in noradrenergic activity.



## **Low dose (less than 15%) CO<sub>2</sub> inhalation in healthy volunteers and patients**

More prolonged (typically 15–20 minutes) inhalation of CO<sub>2</sub> at lower concentration (between 5.0–7.5%) does not frequently result in panic, but reliably induces an experience which resembles the symptoms of GAD, with increased subjective and physiological features of anxiety, but no accompanying changes in cortisol secretion. Studies in healthy volunteers support the use of 20-minute, 7.0–7.5% CO<sub>2</sub> challenge to induce subjective and autonomic responses and neurocognitive changes which resemble the features of generalised anxiety. Increases in heart rate and systolic blood pressure are consistently seen but an increase in diastolic blood pressure is less frequently observed.

Low-dose (7.5%) but prolonged (20 minutes) CO<sub>2</sub> inhalation was first found to induce anxiety in a double-blind, placebo-controlled trial involving healthy volunteers: when compared to normal (placebo) air inhalation, CO<sub>2</sub> inhalation was associated with increased heart rate and blood pressure and heightened subjective anxiety (Bailey et al. 2005). A single-blind, placebo-controlled healthy volunteer study found that when compared to air, 7% CO<sub>2</sub> inhalation increased respiratory rate, minute volume and end-tidal CO<sub>2</sub>, skin conductance and subjective feelings of anxiety: a sub-group of participants who experienced marked anxiety underwent a subsequent identical inhalation with good test-retest repeatability. However, the study findings highlight potential limitations of the model, as 30% of participants were ‘non-responders’, and 10% of participants experienced significant anxiety during (placebo) air inhalation (Poma et al. 2005).

The effect of CO<sub>2</sub> inhalation on attentional biases, which characterize anxiety states, has also been investigated. For example, 20-minute 7.5% CO<sub>2</sub> challenge is associated with performance deficits in an emotional anti-saccade task, similar to those seen in individuals with high levels of generalised trait anxiety (Garner et al. 2011). As twenty minutes of 7.5% CO<sub>2</sub> inhalation has been found to significantly modulate attention, with increased alerting and orienting network function in the Attention Network Task, this suggests that CO<sub>2</sub> challenge facilitates hypervigilance to threat and alters attention network function in a manner consistent with that seen in GAD (Garner et al. 2012).

Inhalation challenges with less than 15% CO<sub>2</sub> provoke significantly more panic attacks in patients with PDA than in healthy controls (Bailey et al. 2011a), but it is uncertain whether altered sensitivity to ‘low dose’ CO<sub>2</sub> inhalation is also seen in patients with GAD. A single-blind, randomised, cross-over design study in medication-free GAD patients which employed a repeated 7.5%, 20-minute inhalation paradigm found CO<sub>2</sub> inhalation increased subjective

anxiety and systolic blood pressure, when compared to air: a qualitative assessment indicated participants' experiences resembled their usual symptoms, more closely for physiological rather than cognitive symptoms (Seddon 2011). The findings should be viewed cautiously given the small sample (n=12) and discontinuation of three participants due to panic responses.

### **Attenuation of CO<sub>2</sub>-induced anxiety by pharmacological interventions**

The effectiveness of psychotropic medication (benzodiazepines, antidepressants, novel compounds) in attenuating CO<sub>2</sub>-evoked anxiety, has been assessed in a number of studies, with variable findings. In general terms, acute benzodiazepine administration reduces subjective CO<sub>2</sub>-provoked anxiety but has little impact on the physiological response. Administration of selective SSRIs, the SNRI venlafaxine, tricyclic antidepressants, and the monoamine oxidase inhibitor toloxatone can all attenuate the panic response to CO<sub>2</sub> challenge (Leibold et al. 2015). Administration of 2mg of lorazepam was found to attenuate subjective anxiety (with no accompanying change in autonomic measures) when compared to placebo in healthy participants undergoing 20 minute 7.5% CO<sub>2</sub> inhalation (Bailey et al. 2007). These findings were replicated when lorazepam was employed as a control in studies using the same inhalation procedure to assess novel anxiolytic compounds (Bailey et al. 2011b; de Oliveira et al. 2012). Both alprazolam (1mg) and the partial benzodiazepine receptor antagonist zolpidem (5mg) attenuated subjective anxiety in healthy volunteers after 20 minutes of 7.5% CO<sub>2</sub> inhalation (Bailey et al. 2009). However, a subsequent double-blind, placebo-controlled cross-over study which investigated dose-response relationships with lorazepam and which used the same experimental paradigm and measures found no attenuation of subjective or autonomic responses (Diaper et al. 2012).

Certain SSRIs and SNRIs are licensed for the treatment of GAD and their effect in attenuating the anxiogenic effects of CO<sub>2</sub> inhalation is a marker of the predictive validity of the model. Investigations in small groups of patients with panic disorder found that treatment with different SSRIs and SNRIs reduced subjective anxiety following 5% and 7% CO<sub>2</sub> challenge, when compared to baseline, pre-treatment inhalation (Gorman et al. 2004). However, a larger study involving 3 minutes of 5% CO<sub>2</sub> in individuals 'at high risk of panic disorder' found that two-week administration of the SSRI escitalopram had no effect on self-report or autonomic indicators of anxiety (Coryell and Rickels 2009). Given that SSRIs typically take 2–4 weeks

to exert notable therapeutic effects in GAD, longer drug administration may be needed to generate valid results.

Studies involving SSRI or SNRI administration in healthy volunteers using a 20-minute 7.5% CO<sub>2</sub> challenge have generated variable findings. Placebo-controlled administration of the SSRI paroxetine for 21 days reduced subjective anxiety (Bailey et al. 2007). A placebo-controlled investigation of three-week administration of the SNRI venlafaxine or the anxiolytic pregabalin found no significant effect on change from baseline to post-treatment ratings of subjective anxiety or autonomic response in the venlafaxine group (Diaper et al. 2013). A two-week randomised double-blind, placebo-controlled study of the SNRI duloxetine in healthy subjects found it had little attenuating effect on subjective anxiety or autonomic arousal following 20 minute, 7.5% CO<sub>2</sub> challenge, though duloxetine administration was associated with improved accuracy in the anti-saccade task and reduction in negative thought intrusions (Pinkney et al. 2014).

As with benzodiazepines, SSRI or SNRI administration has a limited effect on physiological responses to CO<sub>2</sub> challenge, and drugs within the same class may act variably on subjective anxiety, which raises questions about the validity of the model. However, a study involving the beta-blocker propranolol (40mg) found it had no attenuating effect on self-report anxiety in healthy volunteers undergoing 20 minutes of 7.5% CO<sub>2</sub> (Papadopoulos et al. 2010), which accords with its lack of efficacy in anxiety disorders (Gorman et al. 1988; Steenen et al. 2015); and the same study also found the anti-histamine hydroxyzine (25mg) had only limited effects, which accords with its questionable efficacy in relieving symptoms of GAD (Gorman et al. 1988).

### **From current knowledge to potential clinical applications**

The response to CO<sub>2</sub> inhalation could also be useful in predicting the likelihood of response to treatment, but this potential application has not been examined extensively. Investigation of the effects of double 35% CO<sub>2</sub> vital capacity inhalations in a small sample of patients with PDA after 1 hour, 2 weeks and 6 weeks of clonazepam treatment found that when compared to placebo both acute and chronic clonazepam administration reduced objectively rated panic attacks after CO<sub>2</sub> inhalation (Valenca et al. 2002).

Inhalation of air ‘enriched’ with 7.5% CO<sub>2</sub> is an experimental tool for inducing anxiety without features of panic in healthy volunteers, the anxious response being composed of replicable changes in autonomic arousal (increased heart rate and systolic blood pressure),

neurocognitive function (impaired performance in emotional antisaccade and attention control tasks) and subjective experience. The CO<sub>2</sub> inhalation experimental model of anxiety disorders may therefore be useful for signalling the potential efficacy of novel therapeutic agents: and has been utilised in investigations of the CRF<sub>1</sub> receptor antagonist R317573 (Bailey et al. 2011a) and the NK<sub>1</sub> receptor antagonists vestipitant and vofopitant (Poma et al. 2014). The model may be suitable for testing putative anxiolytics (Bailey et al. 2007), and compounds which are found to attenuate CO<sub>2</sub>-induced anxiety have potential clinical relevance. Studies with compounds which target chemosensory mechanisms may be informative in the development of anxiolytics with a novel mechanism of action: for example with the ASIC ion channel antagonist amiloride, which has been found to have neuroprotective effects (Arun et al. 2013); with orexin receptor antagonists, which can attenuate anxiety-like responses to CO<sub>2</sub> challenge in rats (Johnson et al. 2012); and with the carbonic anhydrase inhibitor acetazolamide, which blocks the conversion of CO<sub>2</sub> to carbonic acid and thence to hydrogen and bicarbonate ions (Vollmer et al. 2015).

## **SepAD**

CO<sub>2</sub> hypersensitivity was investigated in adult SepAD because children of adults with PDA experience elevated rates of SepAD and because C-SepAD was found to be associated with adult PDA (Bandelow et al. 2001). Support for this hypothesis comes from a study in which 104 children (aged 9–17 years), of whom 57 had an anxiety disorder, underwent 5% CO<sub>2</sub> inhalation (Pine et al. 1998; Pine et al. 2000). In this study, CO<sub>2</sub> hypersensitivity was clearly present for SepAD, as indicated by: (1) enhanced respiratory rate response during CO<sub>2</sub> breathing; (2) elevated minute ventilation; (3) lower end-tidal CO<sub>2</sub> during room-air breathing. These correlates were also observed – albeit to a much lesser degree – in GAD, and were absent in SAD. Similarly, in a study of 212 offspring from 135 families, abnormal respiratory physiology in response to CO<sub>2</sub> exposure was found in offspring with both SepAD and parental PDA relative to offspring with either of these features alone (Roberson-Nay et al. 2010). Given the common physiological perturbations of PDA and SepAD (i.e. physiological abnormalities, respiratory dysregulation, and reaction to inhaled CO<sub>2</sub>), the specificity of this biological correlate need further confirmatory research data.

# Neurophysiology

## *Electroencephalography (EEG) and event-related potentials (ERP) in wakefulness*

Basal instability of the cortical arousal system was reported in quantitative EEG studies as a common feature of most patients with anxiety disorders (Clark et al. 2009). This manifests as excess of specific EEG frequency bands in the theta (4–8 Hz) and alpha (8–13 Hz) ranges throughout most of the brain areas and beta range (above 13 Hz) especially in frontal and central brain regions. While none of the quantitative electroencephalography (qEEG) alterations are specific for anxiety disorders and can be used as diagnostic tests, they are regarded as related to anxiety symptoms and are targeted e.g. by neurofeedback training (Simkin et al. 2014).

### **PDA**

Studies in patients with PDA showed abnormalities in cortical arousal during waking, sensory gating, and heightened cerebral processing of panic-relevant stimuli, as reflected in elevated CNV and P3 components over frontal regions (Clark et al. 2009).

### **GAD**

Electrophysiological studies in GAD found increases in power, sensory processing deficits, and other alterations (Clark et al. 2009). Generally, sleep EEG (polysomnography) findings in anxiety disorders are in line with findings from wake EEG showing altered EEG-vigilance regulation in these patients. Patients with anxiety disorders typically have prolonged sleep latency, reduced sleep efficiency, shortened total sleep time, decreased amount of slow wave sleep and normal sleep latency. Such sleep pattern is most typical for patients with GAD, and is less expressed in PDA, as long as depressive symptoms are absent.

### **SAD**

In SAD, generally indicate tonic hyperarousal, as reflected in reduced low frequency and increased high frequency EEG power and an elevated PI component were found (Clark et al. 2009).

## **Specific phobias**

There are indications for cortical hypervigilance in specific phobias, with indications of enhanced P3 and contingent negative variation components to phobic stimuli. One study has shown that the P3 amplitude can be normalized following successful behavioural therapy (Clark et al. 2009).

## **PTSD**

Frontal asymmetry belongs to the frequently studied biomarkers in PTSD. It is calculated as a difference in mean alpha band power between the left and right frontal cortex over a time span of several minutes. Relatively greater left frontal activity is regarded as related to appetitive motivation, and lower levels of depression and anxiety in PTSD patients (Meyer et al. 2015). However, this biomarker is not specific for the anxiety disorders, as it has also been reported in depression, premenstrual dysphoric disorder, and schizophrenia. Moreover, in some studies, no deviance in alpha asymmetry from the normative control group was found in anxiety disorders (Gordon et al. 2010).

Patients with PTSD, when compared to controls, were found to have decreased resting-state EEG frontal connectivity, which was significantly correlated with PTSD symptom severity, but also with depressive and increased arousal symptoms (Lee et al. 2014). In a review, significant associations have been described between PTSD symptoms not only for alpha EEG rhythm but also for P200 and P300 ERP components (Lobo et al. 2015). Moreover, alterations of ERP components (N200 and P300 amplitudes) while performing an inhibitory control task (Stop Task) were reported to classify veterans with mild traumatic brain injury associated or not associated with the development of PTSD with high accuracy (Shu et al. 2014).

In PTSD, sleep disturbances shortly after trauma exposure predict the development of PTSD at follow-up assessment, however, the evidence is less clear regarding objective polysomnographic indices (Babson and Feldner 2010).

## **OCD**

Over the past two decades, performance monitoring has been extensively studied using different methodologies, such as source localization and simultaneous EEG, intracerebral recording, magnetoencephalography and EEG-informed fMRI, and valuable results obtained.

Research on ‘performance monitoring’ and ‘error processing’ has been extensively investigated in OCD patients. Clinically, it is recognized that patients with OCD appear to monitor their thoughts and actions most carefully to avoid losing control or committing errors. Theoretically, error processing involves both recognizing that an error has occurred and adjusting future responses. Deficits in either of these abilities could contribute to rigid, repetitive behaviour. Enlarged error signals have been consistently found in patients with OCD (Endrass and Ullsperger 2014). The introduction of specific task paradigms and emotional challenge conditions in such research has been shown to enhance individual differences, which can be more reliable than resting state measurements (Zambrano-Vazquez and Allen 2014).

Error processing is thought to be associated with activity in anterior/posterior medial frontal cortex, anterior insula/operculum, ventrolateral prefrontal cortex, dorsolateral prefrontal cortex, and lateral parietal cortex (Grutzmann et al. 2014). The mid-cingulate cortex is specifically recognized to signal the need for adjustment of cognitive control to prevent subsequent errors (Ullsperger et al. 2014). In particular, the error-related negativity (ERN), a response-locked event-related potential (ERP), is defined as a negative voltage deflection that occurs 50–100 ms after an error or conflict response and is thought to specifically reflect activity of the response monitoring system (Gehring 1990).

Numerous EEG studies have found larger ERN amplitudes in patients with OCD, including adult (Endrass et al. 2008; Endrass et al. 2010; Gehring et al. 2000; Klawohn et al. 2014; Riesel et al. 2011; Riesel et al. 2014; Stern et al. 2010; Xiao et al. 2011) as well as pediatric (Carrasco et al. 2013; Hajcak et al. 2008; Hanna et al. 2012) samples. Enhancement of the ERN in OCD seems to be independent of pharmacologic or psychological interventions (Endrass et al. 2010; Stern et al. 2010) and occurs among all major symptom dimensions (Riesel et al. 2014). Moreover, the same results have been identified in individuals with subclinical OCD symptoms (O'Toole et al. 2012; Santesso et al. 2006) and non-affected first-degree relatives of patients with OCD (Carrasco et al. 2013; Riesel et al. 2011).

Globally, these findings have identified increased ERN amplitudes as a promising candidate vulnerability marker for OCD. However, to date, its sensitivity and specificity it is not clearly defined (Manoach and Agam 2013). For example, some studies have also found an enhanced negativity on *correct* trials (sometimes referred to as the correct-related negativity), suggesting the presence of an overall hyperactivity during response monitoring in people with OCD (Maltby et al. 2005; Ursu et al. 2003). Broadly, amplified error signals in OCD might

reflect hyperactive cortico-striatal circuitry during action monitoring (Agam et al. 2014; Grutzmann et al. 2014). Convergent results suggest the existence of a self-monitoring imbalance involving inhibitory deficits and executive dysfunctions in OCD (Melloni et al. 2012). In this model, the imbalance might be triggered by an excitatory role of the basal ganglia (associated with cognitive or motor actions without volitional control) and inhibitory activity of the OFC as well as excessive monitoring of the ACC to block excitatory impulses. This imbalance would simultaneously interact with the reduced activation of the parietal-dorsolateral prefrontal cortex network, leading to executive dysfunction (Melloni et al. 2012). Further electrophysiological data suggests that the candidate network might be extended and include specific additional regions in the medial frontal cortex involved in performance monitoring, such as anterior insula or the pre-supplementary motor area (Bonini et al. 2014; Grutzmann et al. 2014; Ullsperger et al. 2014); posterior mid-cingulate regions (Agam et al. 2011); and sub-genual anterior cingulate cortex regions, for which increased activity has been found in OCD (Agam et al. 2014). Thus, subjects with OCD might tend to evaluate errors as being disproportionately salient. This would support the theory that inappropriate and exaggerated error signalling leads to a pervasive sense of incompleteness and self-doubt and triggers compulsions to repeat behaviours (Maltby et al. 2005). Other theories hypothesize that the ERN is not only associated with error detection, but may be modulated by the affective significance of an error (Hajcak et al. 2005). Hence, other factors that can potentially characterize the overactive response monitoring observed in individuals with OCD, such as error significance, have been also investigated. However, the results have been equivocal with some studies showing no difference in ERN amplitude between conditions with punishment and no punishment after error in participants with OCD but a significant difference in controls (Endrass et al. 2010); others have found that punishing errors leads to an enhanced ERN and, moreover, that it has long-lasting effect on the ERN (Riesel et al. 2012).

In the analysis of the activity of intracortical EEG sources in patients with OCD with the use of low-resolution electromagnetic tomography (LORETA) and independent component analysis, both methods provided evidence for medial frontal hyperactivation in OCD (Koprivova et al. 2011).

OCD patients present moderate, but significant disturbances of sleep continuity measures but frequently no abnormalities of slow wave sleep or REM sleep. Many of the sleep disturbances were characteristic of depression. Severe OCD symptoms were consistently associated with greater sleep disturbance (Paterson et al. 2013).



### *Other Obsessive-Compulsive-Related Disorders (OC-RDs)*

Electrophysiological studies in other OC-RDs are still scarce. One study has attempted to explore the ERN as a measure of response monitoring capabilities in *trichotillomania* (Roberts et al. 2014). Results reported that individuals with hair pulling symptomatology might have significantly smaller ERNs than the control group, supporting the idea that trichotillomania is distinct from OCD. Smaller ERNs are believed to reflect deficits in error checking that contribute to difficulty monitoring one's own actions, and such results might indicate that individuals with symptoms of trichotillomania have shortfalls in self-monitoring, perhaps related to more impulsive tendencies (Roberts et al. 2014). One other study has used meta-analysis to further characterise the ERN in OCD, and pooled data across studies to examine the ERN in OCD with or without *hoarding* (Mathews et al. 2012). When stratified, OCD showed a significantly enhanced ERN only in response conflict tasks. However, OCD with hoarding showed a marginally larger ERN than OCD without hoarding, but only for probabilistic learning tasks. These results suggest that the abnormal ERN in OCD might also be task-dependent, and that OCD with hoarding might show different ERN activity from OCD without hoarding, perhaps suggesting different pathophysiological mechanisms of error monitoring across these clinical dimensions.

In summary, as neurophysiological examinations belong to the most sensitive tests in psychiatry, many alterations in EEG, ERP or PSG were found in patients with anxiety disorders. While some of these alterations can be used as biomarkers for specific research questions, especially in treatment studies looking at hyperarousal in anxiety disorders, they are not specific and cannot be used as diagnostic tests for anxiety disorders. Moreover, many of reported neurophysiological findings are influenced by depressive symptoms and co-existing pharmacological treatment.

### *Heart rate variability*

Cardiologists have long held the view that a heart rate, which fluctuates over time, in contrast to a heart beating to a strict metronomic rhythm, is a marker of good cardiovascular health. Heart rate variability (HRV), the extent to which the interval between beats varies with time, is reduced in several cardiovascular disorders such as after myocardial infarction (Bigger et al. 1992; Carney et al. 2001), in coronary artery disease (Wennerblom et al. 2000) and in hypertension (Singh et al. 1998) and is a predictor of mortality (Dekker et al. 2000; La Rovere et al. 2003). As will be described in this section, heart rate variability is thought to be closely

linked to the function of the autonomic nervous system and its sympathetic and inhibitory parasympathetic influences.

### **Anxiety, cardiovascular disorders and autonomic dysfunction**

Anxiety disorders are associated with cardiovascular disease (Davies and Allgulander 2013; Roest et al. 2010) and may be a risk factor in sudden cardiac death (Kawachi et al. 1994). The leap from employing HRV as a marker in cardiovascular disorders to anxiety disorders relies on the hypothesis that there may be shared dysfunctions in the autonomic nervous system, which underlie, or at least are measurable in, many disorders in both fields.

#### *PDA*

Taking a specific example, an association of panic attacks or PDA with hypertension has been reported both in clinical samples (Davies et al. 1999) and in population based data (Davies et al. 2012), and the possibility that this association is due to shared autonomic dysfunction has been explored (Davies et al. 2007). Symptoms of autonomic activation, such as racing heart, sweating and flushing are included in diagnostic criteria for PDA. Several authors have suggested that autonomic nervous system dysfunction may be an important aetiological factor in PDA, for instance, Klein (1993) categorised panic attacks into two distinct types; attacks caused by false suffocation alarms and those attributable to autonomic surges or hypothalamus-pituitary-adrenal axis activation.

Esler's group studied norepinephrine and adrenaline release (spillover) from major organs in patients with PDA using invasive methods requiring cannulation of large vessels. Spillover of adrenaline from the heart was significantly greater in patients with PDA than in controls at rest. During panic attacks, whole body adrenaline spillover was markedly increased with proportionally smaller increases in norepinephrine spillover (Wilkinson et al. 1998). This finding supports several studies which report evidence of sympathetic over-reactivity in PDA such as enhanced noradrenergic volatility during clonidine challenge (Coplan et al. 1997) and excess blood pressure overshoot on standing (Coupland et al. 1995). The latter effect was not observed in patients with autonomic failure (Mathias 2002) suggesting that the autonomic nervous system is essential in mediating this response. Others have examined central autonomic system function and reported catecholamine or adrenoceptor function as being altered centrally in PDA (Nutt 1989; Tancer et al. 1993). Esler has demonstrated excess catecholamine spillover in hypertension (Esler et al. 2001) and autonomic dysfunction is now understood to be a core aetiology of what was previously termed 'essential' hypertension.

PDA and hypertension may share a failure of control of sympathetic activation, perhaps through compromise of centres which control the C1-adrenergic cell group in the rostral-ventrolateral medulla, which include the raphe pallidum and ventrolateral periaqueductal grey, the latter under the influence of the pre-frontal cortex (Davies et al. 2007; Johnson et al. 2004).

## **HRV measures**

Heart rate variability allows an estimation of autonomic nervous system input to the heart to be ascertained speedily and non-invasively. There are both parasympathetic (cholinergic) and sympathetic (noradrenergic) influence on the heart. The sympathetic nervous system is linked to mobilisation behaviours, often in response to stressors, which may induce the classic ‘flight or fight response’ requiring cardiac activation, whereas the parasympathetic system, mediated through the vagus nerve, is linked to immobilisation and disengagement (Porges 2001).

Frequency of heart rate fluctuations are decreased when sympathetic tone is increased (Pagani et al. 1984) and with parasympathetic blockade (Akselrod et al. 1985).

The most commonly utilized measures HRV measures are ‘frequency-domain’ and ‘time-domain’ variables. Frequency-domain measures are based on power spectral analysis, which allows detection of low frequency (LF) and high frequency (HF) oscillation. HF oscillation relates to the activity of the parasympathetic system, mainly mediate through the vagus nerve, while LF oscillation is thought to be linked to variation in sympathetic tone. The LF/HF ratio was previously employed as a proxy measure of sympatheto-vagal balance (Pagani et al. 1984), having the advantage of being influenced by change in both sympathetic and parasympathetic nervous system cardiac input but the problem that simultaneous change in both parameters might be undetected.

Time-domain measures of HRV fall into two categories. The first are derived from the differences between adjacent beat intervals, the most frequently used being RMSSD (root mean square of successive differences) and pNN50 (mean occasions per hour where change in consecutive normal sinus (NN) intervals exceeds 50 milliseconds (Ewing et al. 1984)). RMSSD and pNN50 are highly correlated with frequency domain derived HF oscillation (Stein et al. 1994). A second category, derived from observing beat to beat intervals over time, includes SDNN which represents the standard deviation of ‘NN’ intervals (Sztajzel 2004). Since SDNN varies with the total recording time, comparisons between values obtained over widely differing time periods are problematic.

## **HRV – association of frequency domain and time domain measures with anxiety disorders**

While the possibility of HRV being a biomarker in anxiety disorders has been considered for more than a decade (Gorman and Sloan 2000), a systematically organised meta-analysis of the relation of HRV to the presence of anxiety disorders has only recently been published.

Chalmers et al. (2014) identified 36 studies meeting criteria requiring a comparison in HRV outcomes between patients with anxiety disorders and controls. The studies had 2086 participants with anxiety disorders and 2204 controls and employed a variety of methodologies. Recording periods ranged from two minutes to 24 hours and studies used frequency domain measures such as LF and HF, time domain measures or other approaches including detection of respiratory sinus arrhythmia. The authors chose not to extract data on LF/HF ratio given its questionable utility and gave RMSSD preference over other time domain measures.

Across all anxiety disorders, the frequency domain HF oscillation variable, reported in 34 studies, was strongly and significantly associated with having an anxiety disorder. The association of time domain measures, reported in 20 studies, was of borderline significance but became highly significant after exclusion of one outlying study. The LF oscillation variable, reported in 22 studies, was a poor predictor of anxiety disorders. When specific anxiety disorders were considered, PDA featured in the most studies with 24 of the 34 manuscripts having some participants with this disorder, in comparison to 13 for PTSD, five for GAD, four for SAD, two for OCD and one for specific phobia. The meta-analysis revealed that time domain measures were strong predictors of PDA, PTSD, and GAD and weaker but still significant predictors of SAD and specific phobia. HF was strongly associated with GAD and SAD and had weaker but significant relations with PDA and PTSD. Neither measure was associated with OCD. LF was not associated with any of the anxiety disorders. The strength of association of both HF and time domain measures of HRV in generalised anxiety disorder, is of interest for the conceptualization of this disorder. Although both analyses rely on only three studies, the results suggest that despite DSM-IV and DSM-5 excluding clinical features suggestive of autonomic dysfunction from the list of symptoms contributing to the diagnosis, GAD may be associated with autonomic dysfunction after all (Thayer et al. 1996).

## **Response of HRV to treatment and experimental neurotransmitter manipulation**

Treatment of anxiety disorders may be associated with a restoration in HRV, especially when the treatment involves modulation of serotonin. Reduced HRV demonstrated in PDA was reversed by a serotonin promoting antidepressant (Yeragani et al. 1999) but not by nortriptyline, which primarily promotes central norepinephrine transmission (Tucker et al. 1997). However, serotonin-modulating drugs are not essential for improvement in HRV on treating anxiety disorders, as demonstrated by Prasko et al. (2011) who illustrated that cognitive behaviour therapy and SSRIs were equally capable of increasing HRV. In healthy individuals, HRV is reduced during panic provoking challenges but SSRI treatment appears to blunt this response (Agorastos et al. 2015). The involvement of the serotonin system in the neurobiology of anxiety disorders has also been examined using the technique of tryptophan depletion (Hood et al. 2005). When this method is applied in subjects who have recovered from anxiety disorders, depletion is associated with a transient return of anxiety symptoms and exaggerated response to stress challenges (Davies et al. 2006). In one study in remitted patients with depression, HRV was measured before and during tryptophan depletion (Booij et al. 2006). Tryptophan depletion was associated with a significant reduction in HRV (ascertained using both time domain measures and the frequency domain HF measure) although this effect was limited to subjects who had experienced suicidal ideation. Notably, these patients experienced increased anxiety during the tryptophan depletion period.

The therapeutic effect of modulating serotonin in anxiety disorders appears, in the majority of studies, to ameliorate autonomic function as reflected in improving heart rate variability. One exception is a study reporting that CBT alone increased HRV in PDA, but that a CBT/SSRI combination did not (Garakani et al. 2009). Nevertheless, the potential for serotonin to influence autonomic function (and thereby HRV) has a neurobiological basis (Davies et al. 2007), since animal studies suggest that pH-dependent serotonergic neurons projecting to the RVLM may tonically inhibit sympathetic outflow (Richerson et al. 2001). Clinically, the enhanced noradrenergic volatility in PDA described during clonidine challenge was attenuated after successful treatment with selective serotonin reuptake inhibitor (SSRI) antidepressants (Coplan et al. 1997).

## **Utility of HRV as a biomarker**

Heart rate variability, whether ascertained using the frequency-domain measure of HF oscillation or by time domain measures, has advantages over other potential biomarkers of

being non-invasive and easy to administer with valid data being obtainable in a matter of minutes. As such, it has potential use in case detection and in large population-based cohorts. As it is ameliorated by treatments that are effective in anxiety disorders and reduced by neurotransmitter manipulations known to provoke anxiety, it offers the possibility of identification of treatment response.

The proliferation of differing outcome measures is receding in importance as a disadvantage since the frequency domain HF measure, and time domain measures (RMSSD, pNN50 and SDNN) appear to be preferable to LF or the LF/HF ratio. However, several common disorders beyond the realm of anxiety are also associated with reduced HRV, including the cardiovascular disorders discussed earlier, depression, Alzheimer's disease, fibromyalgia and diabetes, and indeed any disorder where autonomic nervous system dysfunction is typically present. This reduces specificity for detection of anxiety disorders. Furthermore, HRV is known to decrease with age (Liao et al. 1995), which may complicate its interpretation. Finally, standard HRV measurements cannot be used in subjects who are not in sinus rhythm (Sztajzel 2004).

In summary, HRV appears to offer a degree of sensitivity but limited specificity in anxiety disorders. Ease of ascertainment and the ability to detect treatment related changes are clear strengths. To exploit its utility fully we await population-based longitudinal studies in larger sample sizes where more invasive approaches may be impractical.

## Neurocognition

### *PDA*

In a review of the literature investigating the neuropsychological disturbances PDA, limited support for impairment in short term memory among individuals with PD was found in some but not all studies. Moreover, the studies did find some evidence for impairment in other areas of cognitive functioning, including executive function, long-term memory, visuospatial or perceptual abilities and working memory (O'Sullivan and Newman 2014). The review included 14 studies (total 439 patients, 510 healthy controls), the majority of which had average to high methodological quality. Studies with a sample size of less than 15 participants per group were excluded.

## GAD

In a study including 112 patients with different anxiety disorders, no differences in neuropsychological functions were found in 7 patients with GAD compared to healthy controls; of course, such a study would only have been powered to detect group differences with massive effect size (Airaksinen et al. 2005). Another study found that performance on executive and non-verbal memory tasks of GAD patients (n=40) was largely worse than in healthy controls (n=31). These cognitive deficits seemed to be more marked in patients taking antidepressants than in drug-naïve patients (Tempesta et al. 2013). However, the study was not randomized with regard to medication intake; therefore, it is problematic to assume a causal relationship between antidepressants and cognitive functioning.

## SAD

Cognitive models of SAD assume that patients with SAD have cognitive biases regarding their interpretation of ambiguous social situations. A systematic review of 30 studies on the neuropsychological performance in SAD (698 patients) revealed that individuals with SAD consistently showed decreased performance on tests of verbal memory functions. In particular, the studies showed decreased performance regarding visual scanning and visuoconstructional ability as well as some indication for verbal memory difficulties (O'Toole and Pedersen 2011). Since this review was published, a study compared 25 subjects with SAD and 25 healthy controls and reported no significant between-group differences, based on a composite analysis of variance test (Sutterby and Bedwell 2012). In *post hoc* tests, patients had worse visual working memory performance than controls, but this finding did not withstand Bonferroni correction. In a subsequent study, SAD (n=42 patients) performed worse than healthy controls (n=42) on processing speed, visuospatial construction, visuospatial memory, verbal learning and word fluence (O'Toole et al. 2015).

## OCD

A plethora of evidence has accumulated showing that behavioural performance during cognitive tests, and related functional activations, are abnormal when OCD patients are probed on domains dependent upon the integrity of fronto-striatal circuitry.

*Response inhibition.* The ability of response inhibition can be measured by means of go/no-go tasks (GNG) and stop signal reaction time tasks (SSRT). Both types of paradigm require the

participants to make a motor response on some trials and to withhold the response on some other trials, with the SSRT being more sophisticated in using stepwise tracking to measure inhibitory control. Deficits in response inhibition have been suggested as a candidate cognitive endophenotype for OCD (n=20 patients versus n=20 controls) (Chamberlain et al. 2007b). Moreover, impaired response inhibition was shown to be associated with reduced grey matter volume in the OFC and right inferior frontal regions, as well as increased grey matter volume in the cingulate, parietal and striatal regions in OCD patients (n=31) and matched-relative groups (n=31), as compared to controls (n=31) (Menzie et al. 2008); and these combined behavioural-structural MRI measures were significantly heritable. Inhibition difficulties were also pinpointed at the functional level, whereby successful inhibition on an SSRT task was associated with greater activation in the supplementary motor area in OCD patients (n=41) and their siblings (n=17), versus controls (n=37) (de Wit et al. 2012). Impaired performance on response inhibition tasks was found to have a moderate effect size (0.49) in a meta-analysis on adult OCD patients as compared with control participants (Abramovitch et al. 2013). This meta-analysis comprised 115 studies (total 3452 patients) overall, although only a subset of these related to response inhibition.

*Cognitive flexibility.* The clinical manifestation of OCD is commonly represented by repetitive compulsive acts that might be linked to impaired cognitive flexibility (Chamberlain et al. 2005). The Intradimensional/Extradimensional set shifting paradigm allows a fine-grained examination of different cognitive processes germane to flexible responding including reversal learning, set formation and the ability to inhibit and shift attention between stimuli. By employing this multiple stage paradigm, it was shown that OCD patients were generally able to form an attentional-set but impaired in their ability to switch their focus to a new, previously irrelevant dimension (extradimensional stage, ED shift) (Chamberlain et al. 2006; Veale et al. 1996; Watkins et al. 2005). Considering that impaired performance was unrelated to symptom severity and present irrespective of treatment, ED deficits might represent a trait marker of the disorder (Chamberlain et al. 2006). More conclusively, non-affected first-degree relatives (n=20) exhibited impairments as well, versus controls (n=20) (Chamberlain et al. 2007b).

Across species, the ability to flexibly adjust behavioural responses in face of negative feedback is subserved by the OFC and can be assessed by reversal learning tasks. As such reversal of responses is normally relatively easy for humans to manage, reversal learning abnormalities are mainly identified using imaging rather than behavioural tests, due to ceiling effects for the latter. Dampened OFC activation during reversal learning was reported in one



fMRI study of OCD patients (n=20), as compared to controls (n=27) (Remijnse et al. 2006). Controlling for the potential confounding effect of comorbid depression, Chamberlain and colleagues (Chamberlain et al. 2008) showed that patients with OCD (n=14) and unaffected relatives (n=12) had extensive clusters of hypo-activation in the lateral OFC, lateral PFC and parietal cortices, versus controls (n=15). Task switching abilities, strongly relying on the cross-talk between basal ganglia and prefrontal cortex (Cools et al. 2004), have separately been assessed in OCD patients. Significantly higher error rates in task-switching trials and reduced activation of dorsolateral prefrontal cortex lateral OFC, ACC and caudate body were observed in 21 OCD patients versus 21 controls (Gu et al. 2008).

*Planning.* Executive planning entails the ability of attaining a goal through intermediate steps, which do not necessarily lead directly to that goal. It is tested by means of the Tower of London task and its variants, for which MRI versions are also often available. Studies in OCD patients revealed lengthened responses times (Nielen and Den Boer 2003; Veale et al. 1996) and, on more difficult task versions, impaired performance (Chamberlain et al. 2007a). Planning deficits have been linked with dorsolateral prefrontal cortex and basal ganglia (caudate, putamen) hypo-activation in OCD patients, in a study conducted in 22 medication-free patients and 22 healthy controls (van den Heuvel et al. 2005). Behavioural impairment – fewer correct responses and increased response times- was also found in unaffected relatives of OCD patients compared with normal participants (Delorme et al. 2007), suggesting that planning deficits constitute a vulnerability measure for OCD.

*Goal-directed system and habit learning.* Convergent evidence from the animal and human literature suggests that fronto-striatal loop circuits mediate the balance between purposeful, goal-directed actions and habitual, automatic behaviours. Considering the literature linking fronto-striatal loops to OCD symptomatology, it was proposed that OCD could be characterised as a disorder of maladaptive habit learning (Rauch et al. 2002). The hypothesis has been formally tested in a series of experiments that led to the conclusion that a defective ‘goal-directed system’ may bias OCD patients to heavily rely on habits (Gillan and Robbins 2014). More specifically, it was first shown using an appetitive instrumental learning task that OCD patients (n=21) were not able to refrain from responding to outcomes no longer associated with reward, as compared to controls (n=30) (Gillan et al. 2011). Similarly, in an aversive context, OCD patients were trained to avoid mildly aversive electrical shocks by performing the correct response to a predictive stimulus. Following a training period, participants were instructed that the cable delivering the shock had been disconnected from one of their wrists. Patients (n=25) on average made significantly more responses to the

stimuli no longer associated with any shock than did controls (n=25) (Gillan et al. 2014). An fMRI-compatible version of the task showed that excessive caudate activity was associated with increased performance of the avoidance habits in 37 OCD patients, compared to 33 healthy comparison subjects (Gillan et al. 2015). The finding that aberrant activation in the caudate nucleus occurred more in patients showing a bias towards the premature development of habits suggested that, in OCD, reliance on repetitive, habit-like behaviours might stem from dysfunction within goal-directed behaviour loci within the dorsal striatum (Yin and Knowlton 2006).

Despite the existence of some discordant findings, deficits related to behavioural inhibition, cognitive flexibility and executive functioning seem to represent core traits of OCD, and hold face validity considering the clinical manifestation of the disorder. Neuropsychological and imaging studies demonstrate that non-affected first-degree relatives show, to some extent, similar abnormalities to patients. On the one hand, these shared findings represent valuable tools for investigating the effect of specific genetic variants on both cognitive and neural substrates and importantly for investigating the disorder across species, possibly leading to better treatment. On the other hand, the similarity between affected and non-affected relatives demonstrates that our understanding of the steps leading from an ‘at risk’ or vulnerable state to the development of ‘state’ OCD is limited; as is our understanding of protective or resilience-related biological factors. Multi-modal investigation, providing convergent evidence and guided by specific theoretical hypotheses, might help to address these issues.

## **Other OC-RDs**

*Trichotillomania* has been associated with impaired stop-signal inhibitory control in multiple studies compared to controls, while set-shifting has generally been reported to be intact (Chamberlain et al. 2006; Odlaug et al. 2014). The sample sizes were 17 patients and 20 controls in the former study; and 12 patients and 14 controls in the latter study.

However, there do appear to be some differences in subtypes: in people with childhood onset trichotillomania (<11 years of age, n=42), the neuropsychological profile appears to be more like OCD; i.e. impaired set-shifting and lesser stop-signal impairments; compared to later onset trichotillomania (n=56) (Odlaug et al. 2012).

Patients with *excoriation* (skin picking) disorder (n=20) showed impaired stop-signal inhibition but intact set-shifting versus controls (n=20) (Odlaug et al., 2010). Impaired response inhibition on a stop-signal task was found in patients with trichotillomania (n=12) and their clinically asymptomatic first-degree relatives (n=10) versus controls (n=14) in a

more recent study, suggesting that it may represent a vulnerability or predisposing factor (Odlaug et al. 2014). In a head-to-head comparison of skin picking disorder (n=31 patients) against trichotillomania (n=39 patients), stop-signal impairments were more marked in the former group (Grant et al. 2011).

As is the case for imaging, cognitive studies in relation to compulsive *hoarding* have mostly been undertaken in the context of other disorders, rather than in 'hoarding disorder' as a discrete entity. One exception to this is a recent study that compared cognition in people with hoarding disorder without OCD (n=22), people with OCD plus hoarding (n=24), and healthy controls (n=28) (Morein-Zamir et al. 2014). Deficits in cognitive flexibility were common to both clinical groups, arguing against hoarding disorder having a distinct neuropsychological profile from that of OCD-hoarding, and highlighting the importance of cognitive rigidity in relation to these two disorders.

There are very few cognitive studies of *body dysmorphic disorder* (BDD). One study found that subjects with BDD exhibited deficits in cognitive flexibility in comparison to controls (Jefferies et al., submitted for publication). Consistent with this proposition, patients with comorbid skin picking disorder and BDD (n=16) had disproportionately impaired set-shifting compared to subjects with non-comorbid skin picking disorder (n=39) (Grant et al. 2015). Other research suggests that individuals with BDD may have abnormalities in visual processing (Feusner et al. 2010). The sample size was 17 patients and 16 controls. In sum, caution is warranted due to the small numbers of studies, but there is some evidence that the grooming disorders (trichotillomania, excoriation disorders) are commonly associated with impaired response inhibition; while hoarding disorder and BDD appear more OCD-like in their neuropsychological profiles.

## PTSD

Research on the neuropsychology of PTSD has identified several neurocognitive deficits that co-occur with the disorder (Everly and Horton 1989; Levy-Gigi et al. 2012; Sachinvala et al. 2000; Vasterling et al. 1998). In one study, subjects with PTSD (n=38), trauma-exposed subjects without PTSD (n=108) and healthy control subjects (n=89) did not differ significantly on a number of neuropsychological tests; however, the study was done in a non-clinical sample of undergraduate students (Twamley et al. 2004). In a double-blind study with 18 PTSD patients, treatment with the SSRI paroxetine resulted in a significant increase in verbal declarative memory function (Fani et al. 2009). It remains unclear whether the memory deficits in PTSD can only be attributed to stress-related alterations. As there is a genetic

vulnerability for developing PTSD, cognitive dysfunctions may have existed before the trauma and may have been, at least in part, the reason why vulnerable individuals develop PTSD after a trauma. Cognitive impairments in PTSD have also been attributed to comorbidity with substance abuse or other psychiatric disorders. However, in a study reporting memory function in rape victims with PTSD (n=15), compared to rape victims without PTSD (n=16), deficits were mild and not attributable to comorbid depression, anxiety, or substance abuse (Jenkins et al. 1998).

One DSM-5 criterion for PTSD is the “inability to remember an important aspect of the traumatic event (typically due to dissociative amnesia)”. One may speculate that dissociative amnesia is associated with the memory impairments generally found in PTSD. However, it is contentious whether the phenomenon of dissociative amnesia exists at all (for a discussion, see McNally, 2007).

## Gender issues

In international epidemiological surveys, the female to male ratio of the prevalence rates of anxiety disorders varied between 1.5:1 and 2.1:1 % (Bandelow and Michaelis 2015).

Psychosocial contributors (e.g. childhood sexual abuse and chronic stressors), but also genetic and neurobiological factors have been discussed as possible causes for the higher prevalence in women. Identification of the causes of gender-specific susceptibility for anxiety disorders may be useful for better understanding the etiology of anxiety disorders in general. It is most likely that higher anxiety susceptibility in women is due to a delicate interplay between psychosocial and neurobiological factors. Hypotheses about the role of gender-specific stressors, and gender differences in the expression of fears warrant further investigation. Sex-specific variance has been identified in numerous neurotransmitter systems. The serotonin system may be of particular importance, as most drugs used in the treatment of anxiety disorders enhance serotonin neurotransmission and alterations in the serotonergic system have been found in anxiety patients relative to healthy controls. It seems likely that female sex hormones are involved, as periods of fluctuating levels of estrogen and progesterone have been linked to increase or decrease of symptomatology in patients with PDA. Moreover, a plausible explanation for the gender-specific risk is a genetic one. For example, in PDA, the COMT and MAO<sub>A</sub> genes have been associated with the higher risk of women to develop PDA (Bandelow and Domschke 2015).

## Discussion

To our knowledge, there has been no comparable consensus initiative that put together all major research lines in the field of biomarkers for anxiety, OCD and PTSD. However, it is a challenge to summarize the incredible amount of findings collected in this paper and the accompanying article (see Part I, (Bandelow et al. submitted)) in a simple way.

First, a change in paradigms has been observed. In the 1980s and 1990s, “wet research” predominated, meaning that blood or CSF samples were taken from patients and healthy controls, either in resting state or after challenge tests with anxiety-provoking agents, e.g. lactate or carbon dioxide. Blood-based biomarkers of treatment response in psychiatric disorders remain in early stages of development and none has demonstrated reliability for predicting pharmacological outcome. Although research efforts in the past decades definitely increased our knowledge of the neurobiological underpinnings of pathological anxiety, we still not have the proof that a certain dysfunction of a neurotransmitter system, e.g. the serotonergic system, is the main cause for anxiety disorders. Still, the most robust evidence for an involvement of serotonin derives from the fact that a large number of drugs that are effective in anxiety disorders, OCD and PTSD have a common denominator, i.e. that they have an impact on serotonergic neurotransmission. Serotonin reuptake inhibition is the main mechanism of action of these antidepressants but there also some drugs that have agonist properties at serotonin receptors. Other medications that can treat anxiety act at the GABA binding site. However, as these binding sites are widespread in the brain and have unspecific inhibitory effects, the efficacy of benzodiazepines in anxiety disorders cannot be taken as evidence that a dysfunction of GABA function is the cause of pathological anxiety.

Since the end of the 1990s, there was a strong shift to neuroimaging and genetic studies – which are summarized in Part I of this consensus paper (Bandelow et al. submitted) –, while the publication output in neurochemistry studies seems to have declined.

Interpreting the abundant number of results of neuroimaging studies in anxiety disorders is a difficult task. The existing studies have found abnormalities in many different regions of the brain, and it is a challenge to synopsise the often contradictory findings in a uniform theory. A problem is the high number of statistical comparisons that are possible, and if the results are not corrected for multiple testing, there is a high chance for false-positive findings. The main methodological problem in most of the studies is the small sample size, making it difficult to reliably separate artefacts from substantiate findings.

Likewise, there is a plethora of genetic studies. In association studies, a large number of candidate genes have been investigated. The only clear result that we can derive from these studies is that anxiety disorders are not based on a single gene but are multigenic, while the contribution of single genes is only small. Genome-wide association studies (GWAS) may be a future possibility to separate relevant findings from findings by chance. Again, correction for multiplicity is crucial, and this again requires larger sample sizes that are often used in genetic research. International cooperation is needed to collect large enough samples for this kind of research. Despite the manifold methodological shortcomings, the neuroimaging and genetics fields are two of the most promising areas of neurobiological research. In the future, neurochemistry, neurophysiology, neuropsychology, neuroimaging, genetics and other fields will have to be integrated in order to elucidate the neurobiological causes of anxiety. There is increasing efforts are made to find reliable biomarkers for diagnostic procedures of for prediction of treatment outcome in anxiety disorders, OCD and PTSD. However, as with research in other mental disorders such as depression, there are still not any biologic or genetic predictor of sufficient clinical utility to inform the selection of specific pharmacological compound for an individual patient, because of low sensitivity and specificity of the suggested biomarkers. Ideally, in the future, we will be possibly be able to diagnose a mental disorder simply by taking a blood test and to chose a personalized medication or psychological treatment for this special patient (precision medicine).

## Achnowledgments

The present work was supported by the Anxiety Disorders Research Network (ADRN) within the European College of Neuropsychopharmacology Network Initiative (ECNP-NI).

Katherina Domschke's work was supported by the German Research Foundation (DFG), Collaborative Research Centre "Fear, Anxiety, Anxiety Disorders" SFB-TRR-58, project C02.

## Statement of Interest

If you do not have any conflict of interest please put "None to declare" in the table. IF YOU DON'T TAKE ANY ACTION, YOUR NAME CANNOT APPEAR IN THE AUTHOR LIST

Name	Conflicts of interest
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Bandelow, Borwin	Research funding: European Commission (FP7), speakers' and/or advisory board for Actelion, Glaxo, Janssen, Lundbeck, Meiji-Seika, Otsuka, Pfizer, Servier.
Baldwin, David	Prof. Baldwin has attended advisory boards for Grunenthal, Eli Lilly, Lundbeck, Pfizer, and Servier. His university has received grants from Lundbeck and Pfizer to support research into anxiety disorders.
Riederer, Peter	None to declare
Abelli, Marianna	None to declare
Bolea-Alamanac, Blanca	None to declare
Bourin, Michel	
Chamberlain, Samuel R.	Dr. Chamberlain consults for Cambridge Cognition
Cinosi, Eduardo	
Davies, Simon	None to declare
Domschke, Katharina	None to declare
Fineberg, Naomi	Dr. Fineberg has received financial support in various forms from the following: Otsuka, Lundbeck, Glaxo-SmithKline, Servier, Cephalon, Astra Zeneca, Jazz Pharmaceuticals, Bristol-Myers Squibb, Novartis, Medical Research Council (UK), National Institute for Health Research (UK), Wellcome Foundation, European College of Neuropsychopharmacology, UK College of Mental Health Pharmacists, British Association for Psychopharmacology, International College of Obsessive-Compulsive Spectrum Disorders, International Society for Behavioural Addiction, World Health Organization, Royal College of Psychiatrists
Grünblatt, Edna	None to declare
Jarema, Marek	Dr. Jarema has been on the speakers' and/or advisory board for Angelini, Janssen, Lilly, Lundbeck, and Servier.
Kim, Yong-Ku	None to declare
Maron, Eduard	None to declare
Masdrakis, Vasileios	None to declare
Mikova, Olya	
Nutt, David	None to declare
Pallanti, Stefano	
Pini, Stefano	
Ströhle, Andreas	Research funding: German Federal Ministry of Education and Research (BMBF), German Research Foundation (DFG), European Commission (FP6), Lundbeck; speaker honoraria: AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Lilly, Lundbeck, Pfizer, Wyeth, UCB. Consultant for Actelion. Educational grants: Stifterverband für die Deutsche Wissenschaft, Berlin Brandenburgische Akademie der Wissenschaften, Boehringer Ingelheim Fonds, Eli Lilly International Foundation, Janssen-Cilag, Pfizer, Lilly
Thibaut, Florence	
Vaghi, Matilde	None to declare
Won, Eunsoo	None to declare
Wedekind, Dirk	
Wichniak, Adam	Dr. Wichniak has been on the speakers' and/or advisory board for Angelini, Janssen, Lundbeck, and Servier.
Wooley, Jade	
Zwanzger, Peter	Speakers' and/or advisory board for Lundbeck, Pfizer, Servier, Aristo, Merz and Hexal

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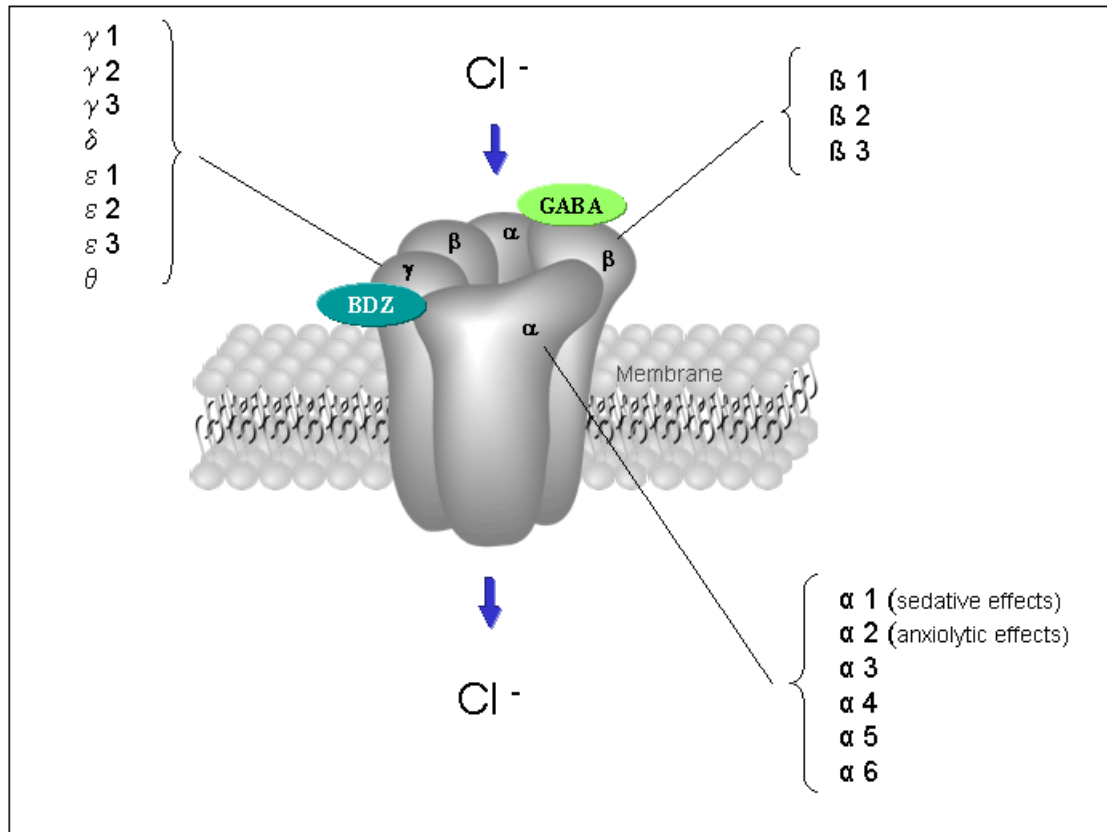
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## Figures

**Figure 1. GABA-A receptor and subunit structure; GABA and benzodiazepine (BZD) binding site**



## Figure 2. Stress-induced interactions between nervous, endocrine and immune systems

The hypothalamus secretes CRH in response to stress, and from the paraventricular nucleus of the hypothalamus. CRH-containing neurons have projections to the locus coeruleus. The locus coeruleus sends direct projections to the sympathetic and parasympathetic preganglionic neurons, increasing sympathetic activity and decreasing parasympathetic activity through the activation of adrenoceptors. In turn, the activation of the sympathetic nervous system stimulates the release of CRH. The products of sympathetic and parasympathetic nervous system activity are NE and E, and ACh, respectively. When stress is prolonged, as in anxiety disorders, the sympathetic nervous system continues to be activated with a lack of parasympathetic counteractivity. As a result, NE and E levels are increased and ACh levels are decreased, which leads to an increased release of pro-inflammatory cytokines from immune cells. Pro-inflammatory cytokines such as TNF, IL1 and IL6 then trigger the activation of the sympathetic nervous system.

CRH, corticotropin-releasing hormone; NE, norepinephrine; E, epinephrine; ACh, acetylcholine, TNF, tumor necrosis factor; IL1, interleukin-1; IL6, interleukin-6; +, stimulation; -, inhibition

